No evidence for increased transmissibility from recurrent mutations in SARS-CoV-2

Lucy van Dorp[1*+]
Damien Richard[2,3*]
Cedric CS. Tan[1]
Liam P. Shaw[4]
Mislav Acman[1]
François Balloux[1+]

[1] UCL Genetics Institute, University College London, London WC1E 6BT, UK
[2] Cirad, UMR PVBMT, F-97410 St Pierre, Réunion, France
[3] Université de la Réunion, UMR PVBMT, F-97490 St Denis, Réunion, France
[4] Nuffield Department of Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK

*contributed equally
+ corresponding; lucy.dorp.12@ucl.ac.uk (Lucy van Dorp) and f.balloux@ucl.ac.uk (François Balloux)

Abstract

The COVID-19 pandemic is caused by the coronavirus SARS-CoV-2, which jumped into the human population in late 2019 from a currently uncharacterised reservoir. Due to this extremely recent association with humans, SARS-CoV-2 may not yet be fully adapted to its human host. This has led to speculations that some lineages of SARS-CoV-2 may be evolving towards higher transmissibility. The most plausible candidate mutations under putative natural selection are those which have emerged repeatedly and independently (homoplasies). Here, we formally test whether any of the recurrent mutations that have been observed in SARS-CoV-2 to date are significantly associated with increased viral transmission. To do so, we developed a phylogenetic index to quantify the relative number of descendants in sister clades with and without a specific allele. We apply this index to a carefully curated set of recurrent mutations identified within a dataset of over 23,000 SARS-CoV-2 genomes isolated from patients worldwide. We do not identify a single recurrent mutation in this set convincingly associated with increased viral transmission. Instead, recurrent SARS-CoV-2 mutations currently in circulation appear to be evolutionary neutral. Recurrent mutations also seem primarily induced by the human immune system via host RNA editing, rather than being signatures of adaption to the novel human host. We find no evidence at this stage for the emergence of more transmissible lineages of SARS-CoV-2 due to recurrent mutations.

Keywords
Betacoronavirus; Homoplasies; Mutation; Phylogenetics; Transmission

Introduction

Severe acute respiratory coronavirus syndrome 2 (SARS-CoV-2), the causative agent of Covid-19, is a positive single-stranded RNA virus that jumped into the human population towards the end of 2019 [1-4] from a yet uncharacterised zoonotic reservoir [5]. Since then, the virus has gradually accumulated mutations leading to patterns of genomic diversity which can be leveraged to inform on the spread of the disease and to identify sites putatively under selection as SARS-CoV-2 may adapt to its new human host. Large-scale efforts from the research community during the ongoing Covid-19 pandemic have resulted in an unprecedented number of SARS-CoV-2 genome assemblies available for downstream analysis. To date (8 June 2020), the Global Initiative on Sharing All Influenza Data (GISAID) [6, 7] repository has nearly 27,000 complete high-quality genome assemblies available. This is being supplemented by increasing raw sequencing data available through the European Bioinformatics Institute (EBI) and NCBI Short Read Archive (SRA), together with data released by specific genome consortiums including COVID-19 Genomics UK (COG-UK) (https://www.cogconsortium.uk/data/). Research groups around the world are continuously monitoring the genomic diversity of SARS-CoV-2, with a focus on the distribution and characterisation of emerging mutations.

Mutations within coronaviruses, and indeed all RNA viruses, can arrive as a result of three processes. First, mutations arise intrinsically as copying errors during viral replication, a process which may be reduced in SARS-CoV-2 relative to other RNA viruses, due to the fact that coronavirus polymerases include a proof-reading mechanism [8, 9]. Second, genomic variability might arise as the result of recombination between two viral lineages co-infecting the same host [10]. Third, mutations can be induced by host RNA editing systems, which form part of natural host immunity [11-13]. While the majority of all mutations are expected to be neutral [14], some may be advantageous or deleterious to the virus. Mutations which are highly deleterious, such as those preventing virus host invasion, will be rapidly purged from the population; mutations that are only slightly deleterious may be retained, if only transiently. Conversely, neutral and in particular advantageous mutations can reach higher frequencies.

Mutations in SARS-CoV-2 have already been scored as putatively adaptive relying on a range of population genetics methods [1, 15-20], and there have been suggestions that some mutations are associated with increased transmission and/or virulence [15, 16, 19]. Early flagging of such adaptive mutations could arguably be useful to control the pandemic. However, distinguishing neutral mutations, whose frequencies have increased through demographic processes, from those directly increasing the virus' transmission can be difficult [21]. The most plausible candidate mutations under putative natural selection are therefore those that have emerged repeatedly and independently within the global viral phylogeny. Such homoplasic sites may arise convergently as a result of the virus responding to adaptive pressures.

Previously, we identified and catalogued homoplasies across SARS-CoV-2 assemblies, of which approximately 200 could be considered as warranting further inspection following stringent filtering [1]. A crucial next step is to test the potential impact of these and other more recently emerged homoplasies on transmission. For a virus, transmission fitness can be considered as a proxy for overall fitness [22, 23]. This allows transmission fitness to be estimated as the relative fraction of descendants produced by an ancestral virion genotype. Such an approach feels warranted, in this case, given the unprecedented number of available SARS-CoV-2 isolates and the lack of strong structure in the global distribution of genetic diversity caused by the large number of independent introduction and transmission events in most densely sampled countries [1]. The fairly homogeneous global distribution pattern of SARS-CoV-2 genetic diversity with 'everything being everywhere' limits the risk that a homoplasic mutation could be

deemed to provide a fitness advantage to its viral carrier simply because it is overrepresented, by chance, in regions of the world more conducive to transmission.

In this work, we make use of a larger, curated alignment comprising 23,090 SARS-CoV-2 assemblies to formally test whether any identified recurrent mutation is involved in altering viral fitness. We find that none of the recurrent SARS-CoV-2 mutations tested are associated with increased viral transmission. Instead, recurrent mutations seem to be primarily induced by host immunity through RNA editing mechanisms, and likely tend to be selectively neutral, with no or only negligible effects on virus transmissibility.

Results

Global diversity of SARS-CoV-2

The global genetic diversity of 23,090 SARS-CoV-2 genome assemblies is presented as a maximum likelihood phylogenetic tree (**Figure 1A)**. No assemblies were found to deviate by more than 29 SNPs from the reference genome, Wuhan-Hu-1, which is consistent with the relatively recent emergence of SARS-CoV-2 towards the latter portion of 2019 [1-5]. We informally estimate the mutation rate over our alignment to 9.6 x 10^{-4} substitutions per site per year, which is consistent with previous rates estimated for SARS-CoV-2 [1-4] (**Figure S1-S2**). This rate also falls in line with those observed in other coronaviruses [24, 25], and is fairly unremarkable relative to other positive single-stranded RNA viruses, which do not have a viral proof-reading mechanism [26, 27].

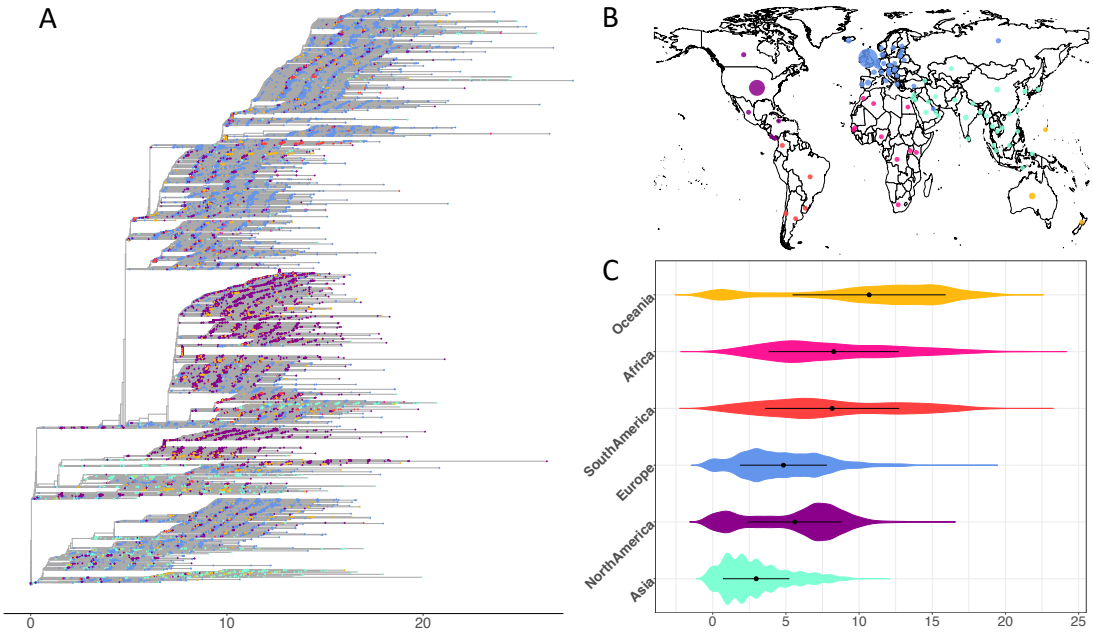

Figure 1 Overview of the global genomic diversity across 23,090 SARS-CoV-2 assemblies (sourced 4 June 2020) coloured as per continental regions**. A.** Maximum Likelihood phylogeny for complete SARS-CoV-2 genomes. **B.** Viral assemblies available from 86 countries. C. Within-continent pairwise genetic distance on a random subsample of 100 assemblies from each continental region. Colours in all three panels represent continents where isolates were collected. Magenta: Africa; Turquoise: Asia; Blue: Europe; Purple: North America; Yellow: Oceania; Orange: South America according to metadata annotations available on GISAID (https://www.gisaid.org) and Table S1.

Genetic diversity in the SARS-CoV-2 population remains moderate with an average pairwise SNP difference across isolates of 9.71 (3-19, 95% CI). This low number of mutations between any two

viruses currently in circulation means that, to date, SARS-CoV-2 can be considered as a single essentially clonal lineage, notwithstanding taxonomic efforts to categorise extant diversity into sublineages [28]. Our dataset includes viruses sequenced from 86 countries (**Figure 1B, Table S1**), with a good temporal coverage (**Figure S1B)**. While some countries are far more densely sampled than others (**Figure 1B**), the emerging picture is that fairly limited geographic structure is observed in the viruses in circulation in any one region. All major clades in the global diversity of SARS-CoV-2 are represented in various regions of the world (**Figure 1A, Figure S3**), and the genomic diversity of SARS-CoV-2 in circulation in different continents is fairly uniform (**Figure 1C**).

Distribution of recurrent mutations

Across the alignment we detect 8,667 variable positions resulting in an observed genomewide ratio of non-synonymous to synonymous substitutions of 1.98 (calculated from **Table S2**). Following masking and homoplasy detection we observe over 3,000 homoplasic positions (3,262 and 3,227 respectively using two different masking criteria), see Methods and **Figures S4-S5, Table S3**. However, recurrent mutations may arise as a result of sequencing or genome assembly artefacts [29]. In line with our previous work ([1]; see Methods) we therefore applied two stringent filtering approaches to delineate sets of well supported homoplasic sites. This resulted in 308 and 300 homoplasic sites in the alignments, respectively (**Figures S4-S5, Table S3**). The current distribution of genomic diversity across the alignment, together with identified homoplasic positions is available as an open access and interactive web-resource at: https://macman123.shinyapps.io/ugi-scov2-alignment-screen/.

As identified by previous studies [30-35], we recover evidence of strong mutational biases across the SARS-CoV-2 genome. A remarkably high proportion of C→U changes was observed relative to other types of SNPs and this pattern was observed at both non-homoplasic and homoplasic sites (**Figures S6-S8**). Additionally, mutations involving cytosines were almost exclusively C→U mutations (98%) and the distributions of *k*-mers for homoplasic sites appeared markedly different compared to that across all variable positions (**Figures S9-S10**). In particular, we observe an enrichment in CCA and TCT 3-mers containing a variable base in their central position, which are known APOBEC targets [36].

Signatures of transmission

In order to test for an association between individual homoplasies and transmission, we defined a novel phylogenetic index designed to quantify the fraction of descendent progeny produced by any ancestral virion having acquired a particular mutation. We term this index the Ratio of Homoplasic Offspring (RoHO). In short, the RoHO index computes the ratio of the number of descendents in sister clades with and without a specific mutation over all independent emergences of a homoplasic allele (shown in red in **Figure 2**). We confirmed that our approach was unbiased (i.e. produced symmetrically distributed RoHO index scores around the Log_{10}(RoHO)=0 expectation for recurrent mutations not associated to transmission) both by analysing simulated nucleotide alignments and discrete traits randomly assigned onto the global SARS-CoV-2 phylogeny (see Methods, **Figure S11**).

We restricted the analysis of the global SARS-CoV-2 phylogeny to homoplasies determined to have arisen at least n=3 times independently. We ignored all homoplasic events where the parent node led to fewer than two descendent tips carying the ancestral allele and two with the derived allele (**Figure 2**). In order to avoid pseudoreplication (i.e. scoring any genome more than once), we also discarded from the RoHO index calculations any homoplasic parent node embedding a secondary homoplasic event involving the same site in the alignment (**Figure 2**).

Ignoring embedding homoplasic parent nodes led to only a marginal loss of statistical power and inclusion of homoplasies carried on embedded nodes yielded similar results (**Figure S11**).

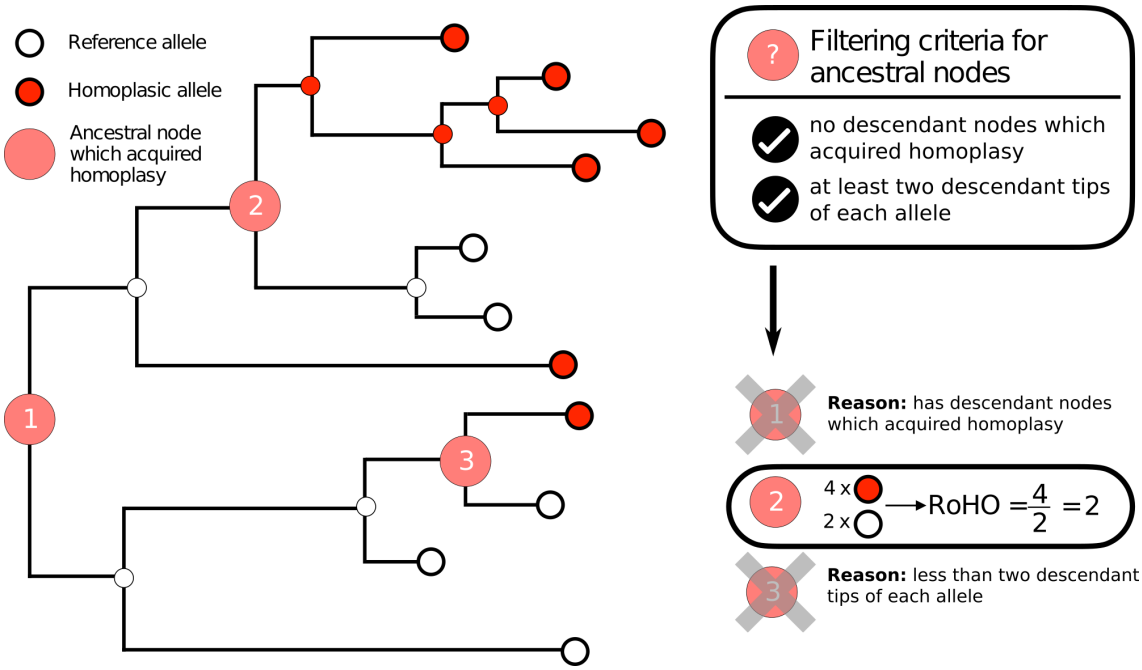

Figure 2 Schematic depicting the rationale behind the Ratio of Homoplasic Offspring (RoHO) score index. White tips correspond to an isolate carrying the reference allele and red tips correspond to the homoplasic allele. This schematic phylogeny comprises three highlighted internal nodes annotated by HomoplasyFinder as corresponding to an ancestor that acquired a homoplasy. Node 3 is not considered because it fails our criterion of having at least two descendant tips carrying either allele. Node 1 is not considered because it includes embedding children nodes themselves annotated as carrying a homoplasic mutation. Node 2 meets our criteria: its RoHO score is 4/2 = 2. In order to consider RoHO score for a homoplasic position, at least n=3 nodes have to satisfy the criteria (not illustrated in the figure).

None of the 80 detected recurrent mutations having emerged independently a minimum of three times were statistically significantly associated with an increase in viral transmission (paired t-test; **Figure 3 and Table S4**). We also did not identify any recurrent mutations statistically significantly associated with reduced viral transmissibility. Instead the entire set of 80 recurrent mutations seem to fit an expectation for neutral evolution with respect to transmissibility, with a mean and median overall Log_{10}RoHO score of -0.002 and and -0.06. Moreover, the distribution of individual site-specific RoHO scores is symmetrically distributed around 0 with 41/80 mean positive values and 39/80 negative ones. To summarise, none of the recurrent mutations in circulation to date in SARS-CoV-2, which we identified as candidates for putative adaptation to its novel human host, shows evidence of being associated to viral transmissibility.

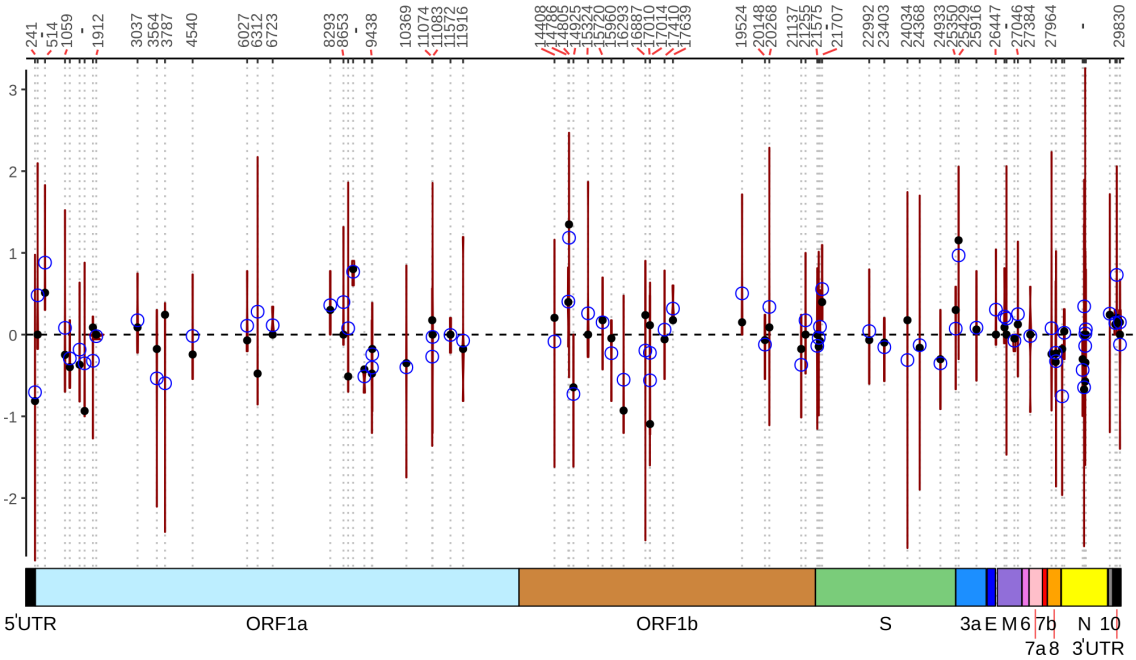

Figure 3. Genome-wide Ratio of Homoplasic Offspring (RoHO) values. Confidence intervals show the $\log_{10}$(RoHO) index for homoplasies that arose in at least three filtered nodes in the Maximum Likelihood phylogeny of 23,090 SARS-CoV-2 isolates. Black dot: median RoHO value; blue circle: mean RoHO value. Number of replicates are provided in **Table S4**. Top scale provides positions of the homoplasies on Wuhan-Hu-1 reference genome and the bottom coloured boxes correspond to encoded ORFs. No homoplasy displayed a RoHO index distribution significantly different from zero (paired t-test, alpha=0.05).

Discussion

In this work, we analysed a dataset of over 23,000 SARS-CoV-2 assemblies sampled across 86 different countries and all major continental regions. Current patterns of genomic diversity highlight multiple introductions in all continents (**Figure 1, Figures S1-S3**) since the host-switch to humans in late 2019 [1-4]. While SARS-CoV-2 still represents a single, essentially clonal lineage, the gradual accumulation of mutations in viral genomes in circulation may offer early clues to adaptation to its novel human host. Across our dataset we identified a total of 8,667 mutations, heavily enriched in C→U transitions, of which we identified 308 strongly supported recurrent mutations (**Table S3, Figures S4-S5**). Employing a newly devised index (Ratio of Homoplasic Offspring; RoHO) to test whether any of these mutations contribute to a change in transmission, we found no mutation was convincingly associated with a significant increase or decrease in transmissibility (**Figures 2-3, Table S4**).

Given the importance of monitoring putative changes in virus transmission, several studies have attempted to associate the presence of particular sets of mutations in SARS-CoV-2 to changes in transmission and virulence [15, 16]. We strongly caution that efforts to determine if any specific mutation contributes to a change in viral phenotype, using solely genomic approaches, relies on the ability to adequately distinguish between changes in allele frequency due to demographic or epidemiological processes, compared to those driven by selection [21]. A convenient and powerful alternative is to focus on sites which have emerged repeatedly independent of the phylogeny (homoplasies), as we do here. While this is obviously restricted to recurrent mutations, it reduces the effect of demographic confounding problems such as founder bias.

A much discussed mutation in the context of demographic confounding is D614G, a nonsynonymous change in the SARS-CoV-2 Spike protein. The D614G mutation emerged early

in the pandemic and is found at high frequency globally, with 16,677 assemblies carrying the derived allele in the data we analysed (**Table S3**). Korber *et al*. suggest that D614G increases transmissibility, and reported experimental evidence consistent with higher viral loads but with no measurable effect on patient infection outcome [15]. Others have suggested an association between D614G and infectivity in human cell lines [19]. However, in line with other recurrent mutations we analysed, D614G (nucleotide position 23,403) does not appear to be associated with significantly increased viral transmission (**Table S4**).

The D614G mutation displays only three independent emergences that qualify for inclusion in our analyses. While this limits our power to detect a statistically significant association with transmissibility, the low number of recurrent mutations leading to the D614G allele suggests that, rather than being a driver a of transmission itself, it arose early and went up in frequency by hitchhiking with one of the deepest branches in the global phylogeny as the SARS-CoV-2 population expanded. In this context, it is of note that D614G is in linkage disequilibrium with two other derived mutations (nucleotide positions 3037 and 11,408) that have experienced highly similar expansions. All three mutations are found in around 72% of the genomes in our global SARS-CoV-2 alignment, and rarely occur in isolation with 99% (16,538/16,677) of genomes with the D614G mutation also carrying the derived alleles at the two other sites. Consistent with our findings that D614G is unlikely to be a large driver of viral transmission, our results support a wider narrative where the vast majority of mutations segregating at reasonably high frequency in SARS-CoV-2 are essentially neutral with respect to viral fitness.

Notably 66% of the detected mutations comprise nonsynonymous changes of which 38% derive from C→U transitions. This high compositional bias, as also detected in other studies [33-35], as well as in other members of the Coronaviridae [30-32], suggests that mutations observed in the SARS-CoV-2 genome are not solely the result of errors by the viral RNA polymerase during virus replication [33, 34]. One possibility is the action of human RNA editing systems which have been implicated in innate and adaptive immunity. These include the AID/APOBEC family of cytidine deaminases which catalyse deamination of cytidine to uridine in RNA or DNA and the ADAR family of adenosine deaminases which catalyse deamination of adenosine to inosine (recognized as a guanosine during translation) in RNA [37, 38].

The exact targets of these host immune RNA editing mechanisms are not fully characterized, but comprise viral nucleotide sequence target motifs whose editing may leave characteristic biases in the viral genome [36, 39, 40]. For example, detectable depletion of the preferred APOBEC3 target dinucleotides sequence TC have been reported in Papillomavirus [41]. In the context of SARS-CoV-2, Simmonds [34] and Di Giorgio et al. [33] both highlight the potential of APOBEC-mediated cytosine deamination as an underlying biological mechanism driving the over-representation of C→U mutations. However, APOBEC3 was shown to result in cytosine deamination but not hypermutation of HCoV-NL63 *in vitro* [42], which may suggest that additional biological processes play a role.

The RoHO index provides an intuitive metric to quantify the association between a given mutation and viral transmission. However, we acknowledge this approach has limitations. First, we have relied on admittedly arbitrary choices concerning the number of minimal observations and nodes required to conduct statistical testing. It seems unlikely this would change our overall conclusions, but results for particular mutations should be considered in light of this caveat. Second, our approach entails some loss of information and therefore statistical power. This is because our motivation to test *independent* occurrences means that we do not handle "nested homoplasies" explicitly: we simply discard them (**Figure 2**), although inclusion of nested homoplasies does not change the overall conclusions (**Figure S11**). Third, our approach is deliberately simple and makes minimal assumptions. More sophisticated approaches for

phylodynamic modelling of viral fitness do exist [23, 43, 44], however, these are not directly portable to SARS-CoV-2 and would be too computationally demanding for a dataset of this size. Fourth, while our approach is undoubtedly more robust to demographic confounding (such as founder bias), it is impossible to completely remove all the sources of bias that come with the use of available public genomes despite stringent quality filtering.

We further acknowledge that the number of SARS-CoV-2 available at this stage of the pandemic, whilst extensive, still provides us only with moderate power to detect statistically significant associations with transmissibility for any individual recurrent mutation (**Figure S12**). Though, the fact that none of the 80 recurrent mutations in the SARS-CoV-2 population we identified as candidates for putative adaptation to its novel human host are statistically significantly associated to transmission suggests that the vast majority of mutations segregating at reasonable frequency are essentially neutral in the context of transmission and viral fitness. This interpretation is supported by the essentially perfect spread of individual RoHO index scores around their expectation under neutral evolution (**Figure 3**).

Our results do not point to any candidate recurrent mutation significantly increasing transmissibility of SARS-CoV-2 at this stage and highlight that the genomic diversity of the global SARS-CoV-2 population is currently still very limited. It is to be expected that SARS-CoV-2 will diverge into phenotypically different lineages as it establishes itself as an endemic human pathogen. However, there is no *a priori* reason to believe that this process will lead to the emergence of any lineage with increased transmission ability in its human host.

Methods

Data acquisition

24,597 SARS-CoV-2 assemblies were downloaded from GISAID on 04/06/2020 selecting only those marked as 'complete', 'low coverage exclude' and 'high coverage only'. To this dataset, all assemblies of total genome length less than 29,700bp were removed, as were any with a fraction of 'N' nucleotides >5%. In addition, all animal isolates strains were removed, including those from bat, pangolin, mink and tiger. All samples flagged by NextStrain as 'exclude' (https://github.com/nextstrain/ncov/blob/master/config/exclude.txt) as of 04/06/2020 were also removed. Five further accessions were also removed from our phylogenetic analyses as they appeared as major outliers following phylogenetic inference despite passing other filtering checks. This left 23,090 assemblies for downstream analysis. A full metadata table and list of acknowledgements is provided in **Table S1**.

Multiple sequence alignment and maximum likelihood tree

All 23,090 assemblies were aligned against the Wuhan-Hu-1 reference genome (GenBank NC_045512.2, GISAID EPI_ISL_402125) using MAFFT [45] implemented in the rapid phylodynamic alignment pipeline provided by Augur (github.com/nextstrain/augur). This resulted in a 29,903 nucleotide alignment. As certain sites in the alignment have been flagged up as putative sequencing errors (http://virological.org/t/issues-with-sars-cov-2-sequencing-data/473, accessed 05/06/2020), we followed two separate masking strategies. The first approach follows NextStrain guidance and masks positions 18,529, 29,849, 29,851 and 29,853 as well as the first 130bp and last 50bp of the alignment. The second masking strategy is designed to test the impact of the inclusion of putative sequencing errors in phylogenetic inference, masking several further sites (n=30) together with the first 55 and last 100 sites of the alignment (the list of sites flagged as 'mask' is available at https://raw.githubusercontent.com/W-L/ProblematicSites_SARS-CoV2/master/problematic_sites_sarsCov2.vcf accessed 04/06/2020) [29]. A complete list of masked positions is provided in **Table S5**. This resulted in two alignments of 23,090 assemblies with 8,667 and 8,645 SNPs respectively.

Resulting alignments were manually inspected in UGene (http://ugene.net). Subsequently, for both alignments, a maximum likelihood phylogenetic tree was built using the Augur tree implementation selecting IQ-TREE as the tree-building method [46]. The resulting phylogenies were viewed and annotated using ggtree [47] (**Figure 1, Figure S1**). Site numbering and genome structure are provided for available annotations (non-overlapping open reading frames) using Wuhan-Hu-1 (NC_045512.2) as reference.

Phylogenetic dating

We informally estimate the substitution rate and time to the most recent common ancestor of both alignments by computing the root-to-tip temporal regression implemented in BactDating [48]. Both alignments exhibit a significant correlation between the genetic distance from the root and the time of sample collection following 10,000 random permutations of sampling date (**Figure S2**).

Homoplasy screen

The resulting maximum likelihood trees were used, together with the input alignment, to rapidly identify recurrent mutations (homoplasies) using HomoplasyFinder [1, 49]. HomoplasyFinder employs the method first described by Fitch [50], providing, for each site, the site specific consistency index and the minimum number of changes invoked on the phylogenetic tree. For this analysis all ambiguous sites in the alignment were set to 'N'. HomoplasyFinder identified a total of 3,262 homoplasies, which were distributed over the SARS-CoV-2 genome (**Figure S4**). For the alternative masking of the alignment, HomoplasyFinder identified a total of 3,227 homoplasies (**Figure S5**).

As previously described, we filtered both sets of identified homoplasies using a set of thresholds attempting to circumvent potential assembly/sequencing errors (filtering scripts are available at https://github.com/liampshaw/CoV-homoplasy-filtering and see reference [1]). Here we only considered homoplasies present in >24 isolates (0.1% of isolates in the dataset), where the proportion of isolates with a homoplasy whose nearest neighbour in tree also had the homoplasy was >0.1, and where the number of submitting and originating laboratories of isolates with the homoplasy was >1. This resulted in 308 filtered sites (300 following a different masking procedure focused on putative sequencing errors) of which 299 overlap (**Table S3**).

In addition, we considered an additional filtering criterion to identify homoplasic sites falling close to homopolymer regions, which may be prone to sequencing error. We defined homopolymer regions as positions on the Wuhan-Hu-1 reference with at least four repeated nucleotides. While homopolymer regions can arise through meaningful biological mechanisms, for example polymerase slippage, such regions have also been implicated in increased error rates for both nanopore [51] and Illumina sequencing [52]. As such, homoplasies detected near these regions (± 1 nt) could have arisen due to sequencing error rather than solely as a result of underlying biological mechanisms. If this were true, we would expect the proportion of homoplasic sites near these regions to be greater than that of homopolymeric positions across the entire genome. We tested this by identifying homopolymer regions using a custom python script (https://github.com/cednotsed/genome_homopolymer_counter) and performing a binomial test on the said proportions. A list of homopolymer regions across the genome is provided in **Table S6**. 27 of the 308 (8.8%) filtered homoplasies were within ± 1 nt of homopolymer regions but this proportion did not differ significantly from that of homopolymeric positions across the reference (9.7%; $p = 0.3252$). As such, we did not exclude homopolymer-associated homoplasies and suggest that these sites are likely to be biologically meaningful.

To determine if systematic biases were introduced in our filtering steps, we performed a principal component analysis (PCA) on the unfiltered list of homoplasies obtained from HomoplasyFinder (n = 3,262). The input space of the PCA included 11 variables, of which eight were dummy-coded reference/variant nucleotides and a further three corresponded to the minimum number of changes on tree, SNP count and consistency index output by HomoplasyFinder. Visualisation of PCA projections (**Figure S8A**) suggested that there was no hidden structure introduced by our homoplasy filtering steps. The first two principle components accounted for 54.8% of the variance and were mostly loaded by the variables encoding the reference and variant nucleotides (**Figure S8B**).

Annotation and characterisation of homoplasic sites

All variable sites across the coding regions of the genomes were identified as synonymous or non-synonymous. This was done by retrieving the amino acid changes corresponding to all SNPs at these positions using a custom Biopython (v.1.76) script (https://github.com/cednotsed/nucleotide_to_AA_parser.git). The ORF coordinates used

(including the ORF1ab ribosomal frameshift site) were obtained from the associated metadata according to Wuhan-Hu-1 (NC_045512.2).

To determine if certain types of SNPs are overrepresented in homoplasic sites, we computed the base count ratios and cumulative frequencies of the different types of SNPs across all SARS-CoV-2 genomes at homoplasic and/or non-homoplasic sites (**Figures S6-S7**). In addition, we identified the sequence context of all variable positions in the genome (± 1 and ± 2 neighbouring bases from these positions) and computed the frequencies of the resultant 3-mers (**Figure S9**) and 5-mers (**Figure S10**).

Quantifying pathogen fitness (transmission)

Under random sampling we expect that a mutation that positively affects a pathogen's transmission fitness will be represented in proportionally more descendent nodes. As such, a pathogen's fitness can be expressed simply as the number of descendent nodes from the direct ancestor of the strain having acquired the mutation, relative to the descendants without the mutation (schematic **Figure 2**). We define this as the Ratio of Homoplasic Offspring (RoHO) index (https://github.com/DamienFr/RoHO).

HomoplasyFinder [49] flags all nodes of a phylogeny corresponding to an ancestor that acquired a homoplasy. We only considered nodes with at least two descending tips carrying either allele and with no children node embedded carrying a subsequent mutation at the same site. For each such node in the tree we counted the number of isolates of each allele and computed the RoHO index. We finally restricted our analysis to homoplasies having at least n = 3 individual RoHO indices (i.e. for which three independent lineages acquired the mutation) and obtained two datasets of varying stringency. Paired t-tests were computed for each homoplasy to test whether RoHO indices were significantly different from zero. To validate the methodology, this analysis was carried out on GISAID data analysed with both masking strategies (**Table S4, Figure S11**).

Assessing RoHO performance

To assess the performance of our RoHO index, we performed a set of simulations designed to test the distribution of RoHO values under a neutral model.

We simulated a 10,000 nucleotide alignment comprising 1000 accessions using the rtree() simulator available in Ape v5.3 [53] and genSeq from the R package PhyTools v0.7-2.0 [54] using a single rate transition matrix multiplied by a rate of $6x10^{-4}$ to approximately match that estimated in [1]. This generated an 8,236 SNP alignment which was run through the tree-building and homoplasy detection algorithms described for the true data; identifying 3,097 homoplasies (pre-filtering). Specifying a minimum of three replicates, a minimum of four descendant offspring with at least two descendant tips of each alleles, we obtained a set of RoHO scores none of which differed significantly from zero (**Figure S11**).

In parallel we tested for any bias in the RoHO scores when a set of randomly generated discrete traits were simulated onto the true maximum likelihood phylogeny. To do so we employed the discrete character simulator rTraitDisc() available through Ape [53] specifying an equilibrium frequency of 1 (i.e. neutrality) and a normalised rate of 0.002 (after dividing branch lengths by the mean edge length). This rate value was manually chosen to approximately reproduce patterns of homoplasies similar to those observed for actual homoplasies. Simulations were repeated for 100 random traits. Considering the discrete simulated traits as variant (putative homoplasic) sites, we again evaluated the RoHO indices (applying filters mentioned previously)

for these 100 neutral traits. One out of the 100 "homoplasies" was significantly different from $\log_{10}$(RoHO) = 0 (paired t-test, alpha=0.05, not corrected for multiple testing).

In all cases, to mitigate the introduction of bias we only considered homoplasies with nodes with at least two tips carrying either allele, in order to avoid 1/n and n/1 comparisons. We further enforced a minimum number of replicates (n=3, **Figure 3, Table S4**). While we discarded homoplasies located on 'embedded nodes' to avoid pseudoreplication (**Figure 2**), we note that including such sites has no impact on our results (**Figure S11**).

In addition we assessed the statistical power to detect significant deviations from neutrality of the RoHO index according to to (i) the number of independent emergences of a homoplasy in the phylogeny and (ii) the imbalance between offspring number for each allele (i.e. fitness differential conferred by the carriage of the derived allele). To do so, we generated 1,000 replicates for each combination of independent emergences (counts) of a homoplasy and corresponding fitness differential values. For each replicate, we drew values for the number of descended tips from the actual homoplasic parent nodes at our 80 candidate mutations sites under putative selection (all 80 pooled). We then probabilistically assigned a state to each tip according to an offspring imbalance (e.g. 10%). We drew replicates until we obtained 1000 for each combination comprising at least two alleles of each type. The proportion of significant paired t-tests for each combination of independent homoplasic parent nodes and fitness differential (10% to 80%) is presented as a heatmap (**Figure S12**).

The statistical power depends primarily on the number of independent emergences (i.e. homoplasic parent nodes) rather than the fitness differential (**Figure S12**). Beneficial alleles have a far higher chance to increase their allele frequency upon introduction than deleterious ones, which are expected to be readily weeded out from the population. Thus, we expect to observe a disproportionally higher number of independent homoplasic parents nodes for beneficial alleles. As such, the RoHO score index is inherently better suited to identify mutations associated to increased transmissibility relative to deleterious ones.

Acknowledgments and Funding

L.v.D and F.B. acknowledge financial support from the Newton Fund UK-China NSFC initiative (grant MR/P007597/1) and the BBSRC (equipment grant BB/R01356X/1). Computational analyses were performed on UCL Computer Science cluster and the South Green bioinformatics platform hosted on the CIRAD HPC cluster. We additionally wish to acknowledge the very large number of scientists in originating and submitting labs who have readily made available SARS-CoV-2 assemblies to the research community. We additionally wish to thank helpful comments on Twitter and bioRxiv, in particularly from Harald Ringbauer, Palle Villesen, Sally Otto and Daniel Falush.

Author Contributions

L.v.D. and F.B. conceived and designed the study; L.v.D., M.A, D.R, L.P.S., C.C.S.T., analysed data and performed computational analyses; L.v.D., and F.B. wrote the paper with inputs from all co-authors.

Competing Interests

The authors have no competing interests to declare.

References

1. van Dorp, L., et al., *Emergence of genomic diversity and recurrent mutations in SARS-CoV-2.* Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases, 2020: p. 104351.
2. Li, X.G., et al., *Transmission dynamics and evolutionary history of 2019-nCoV.* Journal of Medical Virology, 2020. **92**(5): p. 501-511.
3. Giovanetti, M., et al., *The first two cases of 2019-nCoV in Italy: Where they come from?* Journal of Medical Virology, 2020. **92**(5): p. 518-521.
4. Lu, J., et al., *Genomic epidemiology of SARS-CoV-2 in Guangdong Province, China.* medRxiv, 2020: p. 2020.04.01.20047076.
5. Zhou, P., et al., *A pneumonia outbreak associated with a new coronavirus of probable bat origin.* Nature, 2020. **579**(7798): p. 270-+.
6. Elbe, S. and G. Buckland-Merrett, *Data, disease and diplomacy: GISAID's innovative contribution to global health.* Global Challenges, 2017. **1**(1): p. 33-46.
7. Shu, Y.L. and J. McCauley, *GISAID: Global initiative on sharing all influenza data - from vision to reality.* Eurosurveillance, 2017. **22**(13): p. 2-4.
8. Snijder, E.J., et al., *Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage.* Journal of Molecular Biology, 2003. **331**(5): p. 991-1004.
9. Minskaia, E., et al., *Discovery of an RNA virus 3 '-> 5 ' exoribonuclease that is critically involved in coronavirus RNA synthesis.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(13): p. 5108-5113.
10. Lythgoe, K.A., et al., *Shared SARS-CoV-2 diversity suggests localised transmission of minority variants.* bioRxiv, 2020: p. 2020.05.28.118992.
11. Mangeat, B., et al., *Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts.* Nature, 2003. **424**(6944): p. 99-103.
12. Harris, R.S., et al., *DNA determination mediates innate immunity to retroviral infection.* Cell, 2003. **113**(6): p. 803-809.
13. Harris, R.S. and J.P. Dudley, *APOBECs and virus restriction.* Virology, 2015. **479**: p. 131-145.
14. Kimura, M. and T. Ohta, *On the Rate of Molecular Evolution.* Journal of Molecular Evolution, 1971. **1**: p. 1-17.
15. Korber, B., et al., *Spike mutation pipeline reveals the emergence of a more transmissible form of SARS-CoV-2.* bioRxiv, 2020: p. 2020.04.29.069054.
16. Tang, X., et al., *On the origin and continuing evolution of SARS-CoV-2.* National Science Review, 2020.
17. Cagliani, R., et al., *Computational inference of selection underlying the evolution of the novel coronavirus, SARS-CoV-2.* Journal of Virology, 2020: p. JVI.00411-20.
18. Li, X., et al., *Emergence of SARS-CoV-2 through Recombination and Strong Purifying Selection.* bioRxiv, 2020: p. 2020.03.20.000885.
19. Zhang, L., et al., *The D614G mutation in the SARS-CoV-2 spike protein reduces S1 shedding and increases infectivity.* bioRxiv, 2020: p. 2020.06.12.148726.
20. Fountain-Jones, N.M., et al., *Emerging phylogenetic structure of the SARS-CoV-2 pandemic.* bioRxiv, 2020: p. 2020.05.19.103846.

21. MacLean, O.A., et al., *No evidence for distinct types in the evolution of SARS-CoV-2.* Virus Evolution, 2020. **6**(1).
22. Wertheim, J.O., et al., *Transmission fitness of drug-resistant HIV revealed in a surveillance system transmission network.* Virus Evolution, 2017. **3**(1).
23. Kühnert, D., et al., *Quantifying the fitness cost of HIV-1 drug resistance mutations through phylodynamics.* PLOS Pathogens, 2018. **14**(2): p. e1006895.
24. Zhao, Z., et al., *Moderate mutation rate in the SARS coronavirus genome and its implications.* BMC Evolutionary Biology, 2004. **4**(1): p. 21.
25. Dudas, G., et al., *MERS-CoV spillover at the camel-human interface.* eLife, 2018. **7**: p. e31257.
26. Domingo-Calap, P., et al., *An unusually high substitution rate in transplant-associated BK polyomavirus in vivo is further concentrated in HLA-C-bound viral peptides.* Plos Pathogens, 2018. **14**(10): p. 18.
27. Holmes, E.C., et al., *The evolution of Ebola virus: Insights from the 2013-2016 epidemic.* Nature, 2016. **538**(7624): p. 193-200.
28. Rambaut, A., et al., *A dynamic nomenclature proposal for SARS-CoV-2 to assist genomic epidemiology.* bioRxiv, 2020: p. 2020.04.17.046086.
29. De Maio, N., et al., *Issues with SARS-CoV-2 sequencing data.* Virological [Internet], 2020. **5**: p. https://virological.org/t/issues-with-sars-cov-2-sequencing-data/473.
30. Woo, P.C.Y., et al., *Cytosine deamination and selection of CpG suppressed clones are the two major independent biological forces that shape codon usage bias in coronaviruses.* Virology, 2007. **369**(2): p. 431-442.
31. Pyrc, K., et al., *Genome structure and transcriptional regulation of human coronavirus NL63.* Virology Journal, 2004. **1**(1): p. 7.
32. Grigoriev, A., *Mutational patterns correlate with genome organization in SARS and other coronaviruses.* Trends in Genetics, 2004. **20**(3): p. 131-135.
33. Di Giorgio, S., et al., *Evidence for host-dependent RNA editing in the transcriptome of SARS-CoV-2.* bioRxiv, 2020: p. 2020.03.02.973255.
34. Simmonds, P., *Rampant C->U hypermutation in the genomes of SARS-CoV-2 and other coronaviruses – causes and consequences for their short and long evolutionary trajectories.* bioRxiv, 2020: p. 2020.05.01.072330.
35. Rice, A.M., et al., *Evidence for strong mutation bias towards, and selection against, T/U content in SARS-CoV2: implications for attenuated vaccine design.* bioRxiv, 2020: p. 2020.05.11.088112.
36. Salter, J.D. and H.C. Smith, *Modeling the Embrace of a Mutator: APOBEC Selection of Nucleic Acid Ligands.* Trends in Biochemical Sciences, 2018. **43**(8): p. 606-622.
37. Hamilton, C.E., F.N. Papavasiliou, and B.R. Rosenberg, *Diverse functions for DNA and RNA editing in the immune system.* Rna Biology, 2010. **7**(2): p. 220-228.
38. Lamers, M.M., B.G. van den Hoogen, and B.L. Haagmans, *ADAR1: "Editor-in-Chief" of Cytoplasmic Innate Immunity.* Frontiers in Immunology, 2019. **10**: p. 11.
39. Lerner, T., F.N. Papavasiliou, and R. Pecori, *RNA Editors, Cofactors, and mRNA Targets: An Overview of the C-to-U RNA Editing Machinery and Its Implication in Human Disease.* Genes, 2019. **10**(1): p. 19.
40. Salter, J.D., R.P. Bennett, and H.C. Smith, *The APOBEC Protein Family: United by Structure, Divergent in Function.* Trends in Biochemical Sciences, 2016. **41**(7): p. 578-594.

41. Warren, C.J., et al., *Role of the host restriction factor APOBEC3 on papillomavirus evolution.* Virus Evolution, 2015. **1**(1).
42. Milewska, A., et al., *APOBEC3-mediated restriction of RNA virus replication.* Scientific reports, 2018. **8**(1): p. 5960-5960.
43. Rasmussen, D.A. and T. Stadler, *Coupling adaptive molecular evolution to phylodynamics using fitness-dependent birth-death models.* eLife, 2019. **8**: p. e45562.
44. Maddison, W.P., P.E. Midford, and S.P. Otto, *Estimating a Binary Character's Effect on Speciation and Extinction.* Systematic Biology, 2007. **56**(5): p. 701-710.
45. Katoh, K. and D.M. Standley, *MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability.* Molecular Biology and Evolution, 2013. **30**(4): p. 772-780.
46. Minh, B.Q., et al., *IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era.* Molecular Biology and Evolution, 2020. **37**(5): p. 1530-1534.
47. Yu, G.C., et al., *GGTREE: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data.* Methods in Ecology and Evolution, 2017. **8**(1): p. 28-36.
48. Didelot, X., et al., *Bayesian inference of ancestral dates on bacterial phylogenetic trees.* Nucleic Acids Research, 2018. **46**(22): p. 11.
49. Crispell, J., D. Balaz, and S.V. Gordon, *HomoplasyFinder: a simple tool to identify homoplasies on a phylogeny.* Microbial Genomics, 2019. **5**(1): p. 10.
50. Fitch, W.M., *Toward defining course of evolution - minimum change for a specific tree topology.* Systematic Zoology, 1971. **20**(4): p. 406–416.
51. Cretu Stancu, M., et al., *Mapping and phasing of structural variation in patient genomes using nanopore sequencing.* Nature Communications, 2017. **8**(1): p. 1326.
52. Schirmer, M., et al., *Illumina error profiles: resolving fine-scale variation in metagenomic sequencing data.* BMC bioinformatics, 2016. **17**: p. 125-125.
53. Paradis, E. and K. Schliep, *ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R.* Bioinformatics, 2019. **35**(3): p. 1367-4803.
54. Revell, L.J., *phytools: an R package for phylogenetic comparative biology (and other things).* Methods in Ecology and Evolution, 2012. **3**(2): p. 217-223.