TITLE PAGE

Title: THE ORIGIN OF A NEW HUMAN VIRUS: PHYLOGENETIC ANALYSIS OF THE EVOLUTION OF SARS-COV-2

RUNNING TITLE: Phylogenetic analysis and evolution of SARS-CoV-2

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ABSTRACT

Objectives: During the first months of SARS-CoV-2 evolution in a new host, contrasting hypotheses have been proposed about the way the virus has evolved and diversified worldwide. The aim of this study was to perform a comprehensive evolutionary analysis to describe the human outbreak and the evolutionary rate of different genomic regions of SARS-CoV-2.

Methods: The molecular evolution in nine genomic regions of SARS-CoV-2 was analyzed using three different approaches: phylogenetic signal assessment, emergence of amino acid substitutions, and Bayesian evolutionary rate estimation in eight successive fortnights since the virus emergence.

Results: All observed phylogenetic signals were very low and consistent trees were obtained. However, after four months of evolution, it was possible to identify regions revealing an incipient viral lineages formation despite the low phylogenetic signal, since fortnight 3. Finally, the SARS-CoV-2 evolutionary rate for regions nsp3 and S, the ones presenting greater variability, was estimated to range from 1.37 to 2.19 x 10^{-3} substitution/site/year.

Conclusions: In conclusion, results obtained in this work about the variable diversity of crucial viral regions and the determination of the evolutionary rate are consequently decisive to understanding essential feature of viral emergence. In turn, findings may allow identifying the best targets for antiviral treatments and vaccines development.

KEYWORDS: SARS-CoV-2, Phylogeny, Evolution, Evolutionary Rate

1. Introduction

Coronaviruses belong to Coronaviridae family and have a single strand of positive-sense RNA genome of 26 to 32 kb in length (Su et al. 2016). They have been identified in different avian hosts (Cavanagh, 2007, Ismail et al. 2003) as well as in various mammals including bats, mice, dogs, etc. Periodically, new mammalian coronaviruses are identified. In late December 2019, Chinese health authorities identified groups of patients with pneumonia of unknown cause in Wuhan, Hubei Province, China (Zhu et al. 2020). The pathogen, a new coronavirus called SARS-CoV-2 (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020), was identified by local hospitals using a surveillance mechanism for "pneumonia of unknown etiology" (Li et al. 2020a, Li et al. 2020b, Zhu et al. 2020). The pandemic has spread rapidly and, to date, more than 14 million confirmed cases and nearly 600,000 deaths have been reported in just over a six months period (World Health Organization, 2020). This rapid viral spread raises interesting questions about the way its evolution is driven during the pandemic. From the SARS-CoV-2 genome, 16 non-structural proteins (nsp1-16), 4 structural proteins [spike (S), envelope (E), membrane (M) and nucleoprotein (N)], and other proteins essential to complete the replication cycle are translated (Cui et al. 2019, Luk et al. 2019). The large amount of information currently available allows knowing, as never before, the real-time evolution history of a virus since its interspecies jump (Zhou et al. 2020). Most studies published to date have characterized the viral genome and evolution by analyzing a small number of sequences (Benvenuto et al. 2020, Cagliani et al. 2020, Phan, 2020) since processing of complete genomes constitutes an enormous demand of time and resources. Despite this, until now, the viral genomic region providing the most accurate information to characterize SARS-CoV-2, could not be established. This lack of information prevent from investigating its molecular evolution and monitoring biological features affecting the development of antiviral and vaccines. Therefore, the aim of this study

101 was to perform a comprehensive viral evolutionary analysis in order to describe the human
102 outbreak and the molecular evolution rate of different genomic regions of SARS-CoV-2.

2. Materials and Methods

2.1 Datasets

In order to generate a dataset representing different geographic regions and time evolution of the SARS-CoV-2 pandemic from December 24th 2019 to April 17th 2020, data of all the complete genome sequences available at GISAID (https: //www.gisaid.org /) on April 18, 2020 were collected. Data inclusion criteria were: a.- complete genomes, b.- high coverage level, and c.- human hosts only (no other animals, cell culture, or environmental samples). Complete genomes were aligned using MAFFT against the Wuhan-Hu-1 reference genome (NC_045512.2, EPI_ISL_402125). The resulting multiple sequence alignment (dataset 1) was split in nine datasets corresponding to nine coding regions: a.- four structural proteins [envelope (E), nucleocapsid (N), spike (S), Orf3a], b.- four nonstructural proteins (nsp1, nsp3, Orf6, and nsp14), and c.- an unknown function protein (Orf8).

More than six thousand SARS-CoV-2 publicly available nucleotide sequences were downloaded. After data selection according to the inclusion criteria, 1616 SARS-CoV-2 complete genomes were included in dataset 1. Sequences of this dataset 1 came from 55 countries belonging to the five continents as follow: Africa: 39 sequences, Americas: 383 sequences, Asia: 387 sequences, Europe: 686 sequences and Oceania: 121 sequences. After elimination of sequences with indeterminate or ambiguous positions, the number of analyzed sequences for each region were: nsp1, 1608; nsp3, 1511; nsp14, 1550; S, 1488; Orf3a, 1600; E, 1615; Orf6, 1616; Orf8, 1612; and N, 1610. Finally, nucleotide sequences were grouped by fortnight (FN) according to their collection date. Table 1 summarizes the number of sequences per fortnight since the beginning of the pandemic up to FN 8. Dataset 2 was created with the variable sequences of each region analyzed in Dataset 1. Dataset 2 was used for the phylogenetic signal analysis and the Bayesian coalescent trees construction.

2.2 Phylogenetic signal

To determine the phylogenetic signal of each of the nine generated alignments, Likelihood Mapping analyzes were carried out (Strimmer & von Haeseler, 1997), using the Tree Puzzle v5.3 program (Schmidt et al. 2002) and the Quartet puzzling algorithm. This algorithm allowed analyzing the tree topologies that can be completely solved from all possible quartets of the n alignment sequences using maximum likelihood. An alignment with defined tree values greater than 70-80% presents strong support from the statistical point of view (Schmidt et al. 2002). Identical sequences were also removed with ElimDupes (Available at https://www.hiv.lanl.gov/content/sequence/elimdupesv2/elimdupes.html) as they increase computation time and provide no additional information about data phylogeny. The best-fit evolutionary model to each dataset was selected based on the Bayesian Information Criterion obtained with the JModelTest v2.1.10 software (Darriba et al. 2012).

2.3 Analysis of amino acid substitutions

Entropy-One (Available at https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) was used to determining the frequency of amino acids at each position for the nine genomic regions analyzed and evaluating their permanence in the eight investigated fortnights.

2.4 Bayesian coalescence and phylogenetic analysis

To study the relationship between SARS-CoV-2 sequences, nine regions of the virus genome were investigated by Bayesian analyses. Phylogenetic trees were constructed using Bayesian inference with MrBayes v3.2.7a (Ronquist et al. 2012). Each gene was analyzed independently with the same dataset used for the phylogenetic signal analysis so that non-identical sequences were included in the analysis. Analyses were run for five million

generations and sampled every 5000 generations. Convergence of parameters [effective sample size (ESS) ≥ 200, with a 10% burn-in] was verified with Tracer v1.7.1 (Rambaut et al. 2018). Phylogenetic trees were visualized with FigTree v1.4.4.

2.5 Evolutionary rate

The estimation of the nucleotide evolutionary rate was made with the Beast v1.10.4 program package (Suchard et al. 2018). Analyses were run at the CIPRES Science Gateway server (Miller et al. 2010). Three hundred and twelve sequences without indeterminations corresponding to the nsp3 (5835nt) and S (3822nt) genes were randomly selected from dataset 1. The sequences represent all the fortnights and most of the geographical locations sampled until April 17. Temporal calibration was performed by date of sampling. The appropriate evolutionary model was selected as described above for phylogenetic signal analysis. The TIM model of nucleotide substitution was used for nsp3 and, the HKY model of nucleotide substitution for S. The analysis was carried out under a relaxed (uncorrelated lognormal) molecular clock model suggest by Duchene & col. (Duchene et al. 2020) and with an exponential demographic, proper for early viral samples from an outbreak (Grassly & Fraser, 2008). Independent runs were performed for each dataset and a Markov chain Monte Carlo (MCMC) with a length of $1.3x10^9$ steps, sampling every $1.3x10^6$ steps, was set. The convergence of the "meanRate" parameters [effective sample size (ESS) ≥ 200, burn-in 10%] was verified with Tracer v1.7.1 (Rambaut et al. 2018). Additionally, in order to verify the obtained results, 15 independent replicates of the analysis were performed with the time calibration information (date of sampling) randomized as described by Rieux & Khatchikian, 2017 (Rieux & Khatchikian, 2017). Finally, the obtained parameters for real data and the randomized replicates were compared.

3. Results

3.1 Phylogenetic signal

Using bioinformatics tools, a phylogenetic signal study was carried out in order to identify the most informative SARS-CoV-2 genomic regions. The likelihood mapping analysis showed that most genes has very poor phylogenetic signal with high values in central region which represents the area of unresolved quartets (Figure 1). Accordingly, genes could be separated into three groups. A group with little or no phylogenetic signal (E, Orf6, Orf8, nsp1, and nsp14), a second group with low phylogenetic signal (Orf3a and N), and a last group with relatively more phylogenetic signal (S and nsp3) but still low to be considered a robust one (unresolved quartets >40%).

3.2 Analysis of amino acid substitutions

The analysis of amino acids substitutions by fortnights was useful to study the viral evolutionary dynamics in the context of the beginning of the pandemic. By analyzing different time periods amino acid sequences, changes were observed in 5 out of 9 regions and only in 14 out of the 4975 (0.28%) evaluated residues. In most of the regions, except nsp1, nsp14, E, and Orf6, 2 to 6 amino acids were selected since FN3 and remain unchanged until the end of the follow up period (Table 2). Particularly, in Orf8 region, early selection of two amino acid substitutions (V62L and S84L) was observed from FN2. On the other hand, in the S region, the D614G substitution started with less than 2% in FN3 and FN4 and reached 88% in the last fortnight. In a similar way, the Q57H (Orf3a) substitution went from 6% to 34% while S84L start to be selected in FN2 and reached 94% by FN8. The R203K and G204R substitutions of the N region was selected in FN4 and increased their population proportion with values greater than 20% towards the end of the follow up period. Moreover, selection of a great number of sporadic substitutions remaining in the population for a short period (1-3 fortnights) was

observed in the nine analyzed regions. Indeed, 333 (6.83%) of the analyzed positions presented at least one substitution throughout the eight fortnights. Table 3 summarizes the number of variable positions, number of mutations, and number of sequences with mutations by region.

3.3 Bayesian coalescence analysis

In this study, trees were performed by Bayesian analysis instead of by distance, likelihood, or parsimony methods. Consistently with the phylogenetic signal analysis, trees for nsp1, E, and Orf6 showed a star-like topology. Nevertheless, different proportions of clades formation could be observed in trees of Orf8, nsp14, Orf3a, N, S, and nsp3 regions (Figure 2). Finally, from mentioned regions, nsp3 and S showed a better clade constitution. This analysis allowed to differentiate regions presenting a diversification process (nsp3, nsp14, Orf3a, S, Orf8, and N) from those that even after four months showed an incipient one (nsp1, E, and Orf6). Furthermore, this nucleotide analysis is complemented by the previous study of amino acid variations in each region. However, it is important to note that due to the low phylogenetic signal observed for each region, results can only be considered as preliminary.

3.4 Evolutionary rate

Nsp3 and S sequences were selected to perform the evolutionary rate analysis since both regions provided the best phylogenetic information among studied regions. The observed evolutionary rate for nsp3 protein of SARS-CoV-2 was estimated to be 1.37 $x10^{-3}$ (ESS 782) nucleotide substitutions per site per year (s/s/y) (95% HPD interval 9.16 $x10^{-4}$ to 1.91 $x10^{-3}$). On the other hand, the corresponding figures for S were estimated to be 2.19 $x10^{-3}$ (ESS 383) nucleotide s/s/y (95% HPD interval 3.19 $x10^{-3}$ to 1.29 $x10^{-3}$). In both genomic regions, date-randomization analyses showed no overlapping between the 95% HPD substitution-rate

228 intervals obtained from real data and from date-randomized datasets. This fact suggests that
229 the original dataset has enough temporal signal to perform analyses with temporal calibration
230 based on tip-dates (Figure 3).

4. Discussion

The phylogenetic characterization of an emerging virus is crucial to understand the way the virus and the pandemic will evolve. Thereby, a detailed study of the SARS CoV-2 genome allows, on the one hand, to contribute to the knowledge of viral diversity in order to detect the most suitable regions to be used as antivirals or vaccines targets. On the other hand, the large amount of information that is continuously generated, is allowing studying the SARS CoV-2 genome and describing a new viral real time evolution like never before.

In the present study, the molecular evolution and viral lineages of SARS-CoV-2 in nine genomic regions, during eight successive fortnights, was analyzed using three different approaches: phylogenetic signal assessment, emergence of amino acid substitutions, and Bayesian evolutionary rate estimation. In this context, the observed phylogenetic signals of nine coding regions were very low and the obtained trees were consistent with this finding, showing star-like topologies in some viral regions (nsp1, E, and Orf6). However, after a four months evolution period, it was possible to identify regions (nsp3, S, Orf3a, Orf8, and N) revealing an incipient formation of viral lineages, despite the phylogenetic signal, both at the nucleotide and amino acid levels from FN3. Based on these findings, the SARS-CoV-2 evolutionary rate was estimated, for the first time, for the two regions showing higher variability (S and nsp3).

As regards the phylogenetic signal, several simulation studies has proven that for a set of sequences to be considered robust, the central and lateral areas representing the unresolved quartets, must not be greater than 40% (Strimmer & von Haeseler, 1997). In this regard, none of the nine analyzed regions met this requirement. Three regions (E, nsp1, and Orf6) presented values of 100% unresolved quartets. Most regions (nsp14, Orf3a, Orf8, and N) reached values higher than 85%. Only in regions nsp3 and S the number of unresolved quartets dropped to ~ 60%. Thus, despite being a virus with an RNA genome, the short time

elapsed since its emergence, and possibly genetic restrictions have led to a constrained evolution of SARS-CoV-2 in these months. For this reason, it is expected that trees generated from SARS-CoV-2 partial sequences in the first months of the pandemic are unreliable for defining clades. Therefore, they should be analyzed with great caution.

Since Bayesian analysis allows to infer phylogenetic patterns from tree distributions, it represents a more reliable tool to compare different evolutionary behaviors. Bayesian analysis helps to obtain a tree topology that is closer to reality in the current conditions of SARS-CoV-2 pandemic (Drummond et al. 2006). The phylogenetic analysis for nsp1, E, and Orf6 regions confirmed the star-like topologies in accordance to a lower diversification of these regions using the sequences available up to FN8 (Figure 2). Trees generated from nsp14 and Orf8 are at an intermediate point, where the formation of small clusters can be observed. In fact, a mutation at position 28,144 (Orf8: C251T, S84L) has been proposed as a possible marker for viral classification (Tang et al. 2020, Yin, 2020). Finally, trees obtained from regions Orf3a, N, nsp3, and S showed the best clade formation. Indeed, in the most variable regions nsp3 and S, it can be clearly seen that sequences are separated into two large groups. Despite the aforementioned for the nsp3 and S regions, even clusters with very high support values should be taken with precaution and longer periods should be considered to obtain more accurate phylogeny data. However, even when data are not the most accurate to study the spread or clade formation (Mavian et al. 2020, Sanchez-Pacheco et al. 2020), they provide a good representation of the way the virus is evolving.

The analysis of amino acids frequencies allowed identifying different degree of region conservation throughout the viral genome as a consequence of positive and negative pressures. In particular, nsp3, S, Orf8, and N showed some substitutions in high frequencies. This would indicate, as other authors previously report, the frequent circulation of polymorphisms due to significant positive pressure (Cagliani et al. 2020, Issa et al. 2020, Tang

et al. 2020). Additionally, since S and N are among candidates to be used in the formulation of vaccines and antibody treatment, it will be important to monitor these substitutions in different geographic regions in order to improve treatment and vaccination efficacy (Ahmed et al. 2020, Callaway, 2020, Koyama et al. 2020). Contrarily, in regions nsp1, nsp14, E, and Orf6, no substitutions were selected and lasted during the first 4 months of the pandemic. This would suggest that these are regions with constraints to change due to the great negative selection pressure, as it has been recently reported (Cagliani et al. 2020).

In the present study, the evolutionary rate for SARS-CoV-2 genes was estimated by analyzing a large number of sequences, which were carefully curated and had a good temporal and spatial structure. Additionally, the most phylogenetically informative regions of the genome (nsp3 and S) were used for analysis, reinforcing the results confidence. Previous studies on SARS-CoV-2 have reported similar data ranging from 1.79 x 10^{-3} to 6.58 x 10^{-3} s/s/y for the complete genome (Giovanetti et al. 2020, Li et al. 2020a). However, in both articles, small datasets of complete genomes were used (N=32 and 54, respectively). As studies were performed early in the outbreak and due to datasets temporal structure, analysis could have led to less precise estimates of the evolutionary rate (Duchene et al. 2020). On the other hand, another study analyzing 7,666 sequences has obtained different results with a remarkably low evolutionary rate (6 x10^{-4} nucleotide/genome/year) (van Dorp et al. 2020). Additionally, tests randomizing the dates of nsp3 and S datasets were carried out; they showed that these partial genomic regions have enough temporal signal. In this context, our results (range from 1.37 to 2.19 x 10^{-3} s/s/y) are in close agreement with those published for SARS-CoV genome, which have been estimated between 0.80 to 3.01 x10^{-3} s/s/y (The Chinese SARS Molecular Epidemiology Consortium, 2004, Vega et al. 2004, Zhao et al. 2004). Moreover, our values are in the same magnitude order as other RNA viruses (Sanjuán, 2012). Even though we

should be cautious with these results interpretation, the date-randomization analysis indicated a robust temporal signal.

Despite limitations of the evolutionary study of an emerging virus, where the selection pressures are still low and therefore its variability is also low, this work has a great strength: it lies on the extremely careful selection of a big sequence dataset to be analyze. First, it was considered selected sequences to have a good temporal signal and spatial (geographic) structure. Secondly, much attention was paid to the elimination of sequences with low coverage and indeterminacies that could generate a noise for the phylogenetic analysis of a virus that is beginning to evolve in a new host.

The appearance of a new virus means an adaptation challenge. The SARS-CoV-2 overcome the spill stage and shows a significantly higher spread than SARS-CoV and MERS-CoV, thus becoming itself the most important pandemic of the century. In this context, the results obtained in this work about the variable diversity of nine crucial viral regions and the determination of the evolutionary rate, are consequently decisive to understanding essential feature of viral emergence. Nevertheless, monitoring SARS-CoV-2 population will be required to determine the evolutionary course of new mutations as well as to understand the way they affect viral fitness in human hosts.

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458 **Table 1.** Number of SARS-CoV-2 sequences by fortnight (Temporal structure)

Fortnight	Date	Median of analyzed sequences (Q1-Q3)
FN1	12/24/2019 to 12/31/2019	15
FN2	01/01/2020 to 01/15/2020	19
FN3	01/16/2020 to 01/31/2020	145 (136-145.5)
FN4	02/01/2020 to 02/15/2020	119 (113-120)
FN5	02/16/2020 to 03/02/2020	258 (247-259)
FN6	03/03/2020 to 03/17/2020	403 (390-406)
FN7	03/18/2020 to 04/01/2020	447 (416-450)
FN8	04/02/2020 to 04/17/2020	199 (197-201)
TOTAL		**1488 to 1616**

459 FN: Fortnight; Q1=quartile 1, Q3=quartile 3. The total number of sequences is variable depending on
460 the analyzed region (nsp1, 1608; nsp3, 1511; nsp14, 1550; S, 1488; Orf3a, 1600; E, 1615; Orf6, 1616;
461 Orf8, 1612; and N, 1610)
462

463 **Table 2.** Amino acids selected by region and fortnight. The number indicates the amino acids
464 location in its protein.

Region	Amino acid substitution	Amino acid percentage by FN							
		FN1	FN2	FN3	FN4	FN5	FN6	FN7	FN8
nsp3	A58T	0	0	0	1.0	6.0	3.0	3.0	2.5
	P135L	0	0	0.8	0	0	1.5	0.5	2.5
S	D614G	0	0	1.5	1.8	37.0	64.0	75.0	88.0
Orf3a	Q75H	0	0	0	0	6.0	22.0	23.0	34.0
	G196V	0	0	0	0	0.8	4.0	0.9	0.5
	G251V	0	0	8.0	24.0	8.0	9.0	10.0	3.0
Orf8	V62L	0	5.0	1.0	3.3	0.0	1.5	1.3	3.0
	S84L	0	58.0	63.0	79.0	79.0	82.0	93.0	94.0
N	P13L	0	0	0	0	1.0	1.0	2.5	0.5
	S197L	0	0	0	0	1.1	5.0	0.9	0.5
	S202N	0	0	3.5	4.2	0	0.5	2.2	2.5
	R203K	0	0	0	0	17.0	19.0	24.0	23.0
	G204R	0	0	0	0	17.0	19.0	24.0	23.0
	I292T	0	0	0	0	2.0	0.2	0.2	0.5

465 Only regions where amino acid change was selected and remained until the last analyzed fortnight are
466 shown. FN: Fortnight; aa: amino acid
467
468

Table 3. Number of variable positions, number of mutations, and number of sequences with mutation by region

Region	Nº of variable aa positions (%)	Nº of aa substitutions	Nº of sequences with aa substitutions (%)
nsp1 (180aa)	3 (1.7)	37	37 (2.4)
nsp3 (1945aa)	158 (8.1)	322	294 (19.3)
nsp14 (527aa)	6 (1.4)	83	83 (5.5)
S (1273aa)	76 (5.9)	1013	904 (59.4)
Orf3a (275aa)	11 (4)	491	468 (30.7)
E (75aa)	5 (6.7)	6	6 (0.4)
Orf6 (60aa)	7 (11.6)	9	9 (0.6)
Orf8 (121aa)	14 (11.6)	312	288 (18.9)
N (419aa)	53 (12.6)	760	470 (30.9)
Total (4875aa)	333 (6.8)	3033	-

aa: amino acid

Figure legends

Figure 1: Phylogenetic signal for SARS-CoV-2 datasets. Presence of phylogenetic signal was evaluated by likelihood mapping, unresolved quartets (center) and partly resolved quartets (edges) for genomes available on April 17 for the nine analyzed regions: nsp1 (29 sequences), nsp3 (225 sequences), nsp14 (65 sequences), S (183 sequences), Orf3a (74 sequences), E (11 sequences), Orf6 (12 sequences), Orf8 (23 sequences), and N (113 sequences). Presence of strong phylogenetic signal (<40% unresolved quartets) was not reached for any region.

Figure 2: Bayesian trees of 29 sequences of nsp1 (540nt), 225 sequences of nsp3 (5835nt), 65 sequences of nsp14 (1581nt), 183 sequences of S (3822nt), 74 sequences of Orf3a (828nt), 11 sequences of E (228nt), 12 sequences of Orf6 (186nt), 23 sequences of Orf8 (366nt), and113 sequences of N (1260nt). Scale bar represents substitutions per site.

Figure 3: Comparison of the evolutionary rates estimated using BEAST for the original dataset and the date-randomized datasets (312 sequences). This analysis was performed for regions nsp3 (5835nt) and S (3822nt). s.s.y = substitutions/site/year.

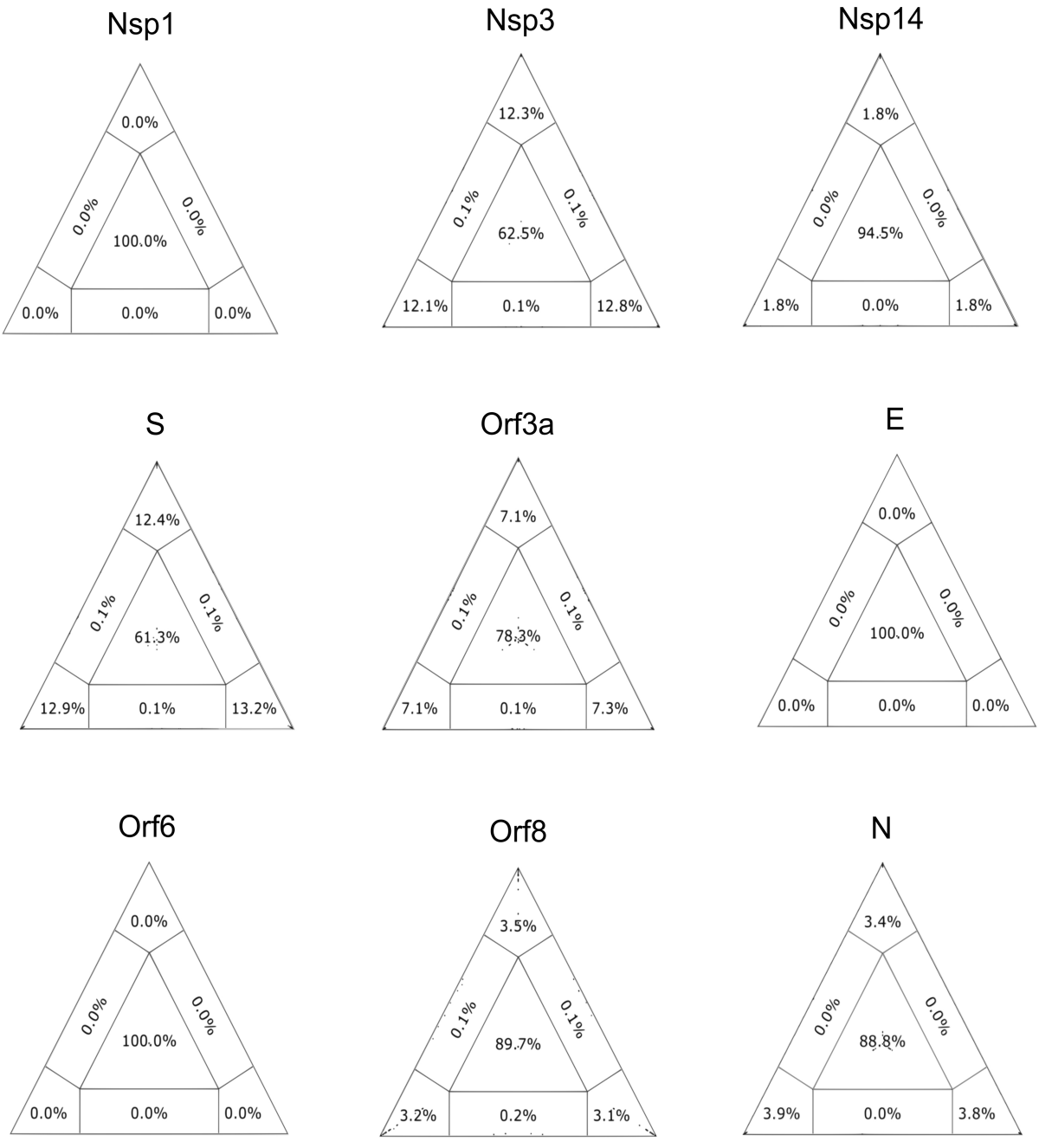

Figure 1

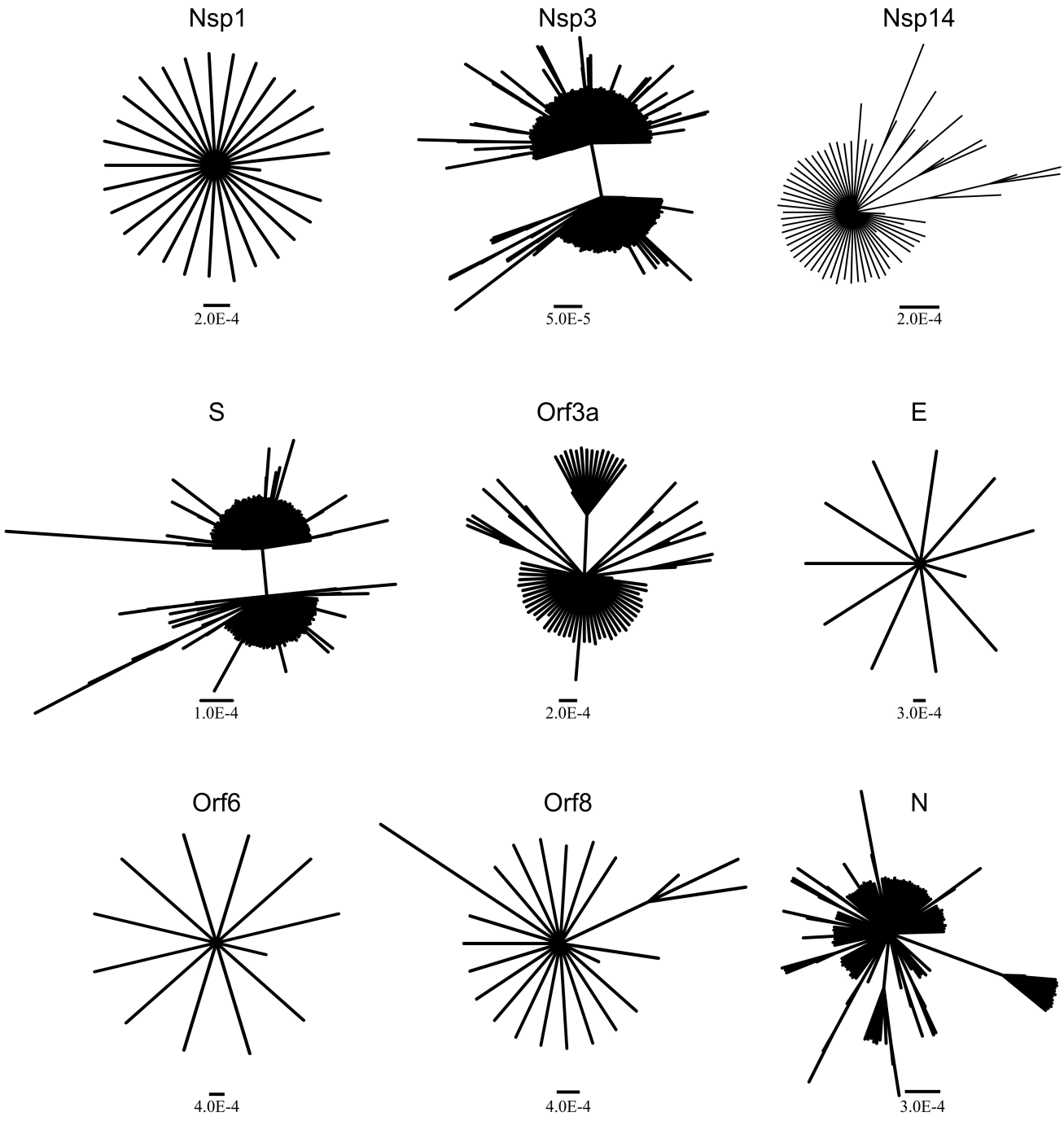

Figure 2

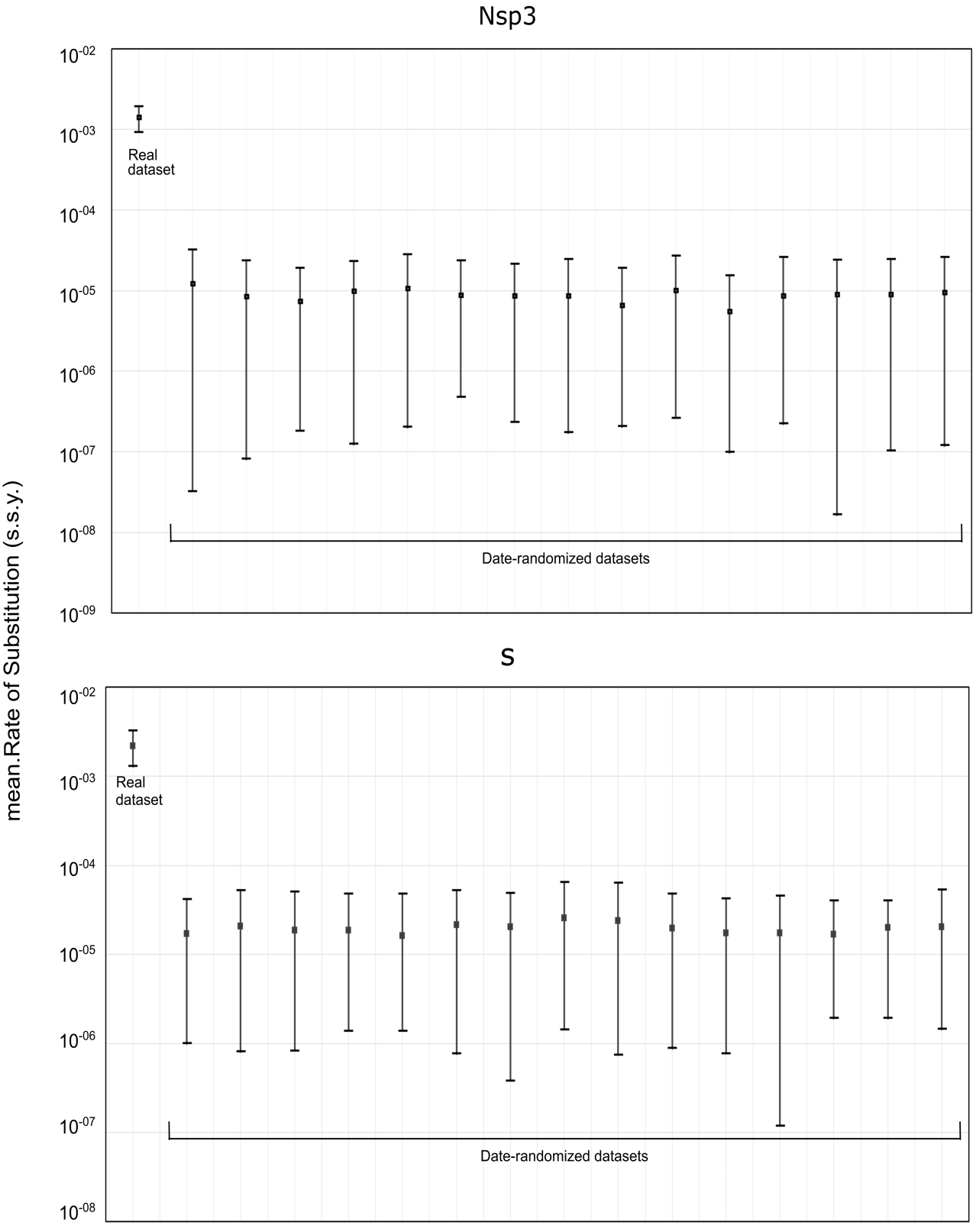

Figure 3