Sequence evidence that the D614G clade of SARS-CoV-2 was already circulating in northern Italy in the fall of 2019

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Abstract

Elucidating ancestor-descendant relationships of viral lineages is crucial for addressing the question of when and where a virulent viral strain originated. The D614G clade, with an Aspartate-614 to Glycine (D614G) mutation, includes all recent variants of SARS-CoV-2 and tend to be more infectious than the viral strains isolated in Wuhan. The D614G clade is characterized by TTTG at four nucleotide sites (sites 241, 3037, 14408 and 23403 following the reference genome NC_045512), in contrast to CCCA shared among early SARS-CoV-2 genomes sampled in China and those that can be traced to China. It was believed that the TTTG lineage descended from the early viral CCCA lineages. A set of SARS-CoV-2 sequences collected from Sept. 12 to Dec. 18, 2019, in Lombardy, Milan and Turin in Italy provided, for the first time, strong evidence that the D614G/TTTG lineage has already been circulating in Italy in 2019. I discussed extensively the controversies arising from this set of early SARS-CoV-2 sequences.

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Two key questions are asked following a viral outbreak, i.e., when and where the zoonotic or lab-leak event occurred. The "When" question is typically addressed by estimating the viral evolutionary rate and dating the most recent common ancestor (MRCA) of representative viral strains [1][2][3][4][5][6]. The "Where" question is approximated by the location where the earliest viral lineage was sampled [5][6][7]. Obviously, such large-scale analyses are better performed with full-length high-quality viral genomes than short sequence fragments. For SARS-CoV-2, there are currently more than 13 million genomic sequences. Thus, there seems no shortage of data for any large-scale data analysis. Indeed, the MRCA of SARS-CoV-2 genomes have been dated to the summer of 2019 with a gigantic tree of 455,251 leaves [6].

One major shortcoming of using full-length high-quality SARS-CoV-2 genomes is that these genomes are sampled

after the viral outbreak in Wuhan in late December 2019, when SARS-CoV-2 has already spread globally. SARS-CoV-2 samples collected before the viral outbreak are present in GenBank only as short sequence fragments. However, these sequence fragments provide critical insights into the origin and early evolution of the D614G lineage which was first detected in China and Germany in January 2020 [8].

The D614G clade differs from the early Wuhan strains at four nucleotide sites (sites 241, 3037, 14408 and 23403 following the reference genome NC_045512) [8][9]. The D614G strain has TTTG at these four sites, in contrast to CCCA shared among early SARS-CoV-2 genomes sampled in Wuhan. It was believed that the D614G/TTTG lineage descends from the CCCA lineage. However, sequence data from early SARS-CoV-2 samples collected in northern Italy (Lombardy, Milan and Turin regions) from Sept. 12 to Dec. 18, 2019, provided strong evidence that the D614G/TTTG lineage was already circulating in northern Italy in the fall of 2019.

Thirteen of these Italy-derived samples of SARS-CoV-2 were partially sequenced and deposited in GenBank [10][11] (Table 1). These sequence fragments vary in length from 209 nt to 778 nt and do not cover the entire SARS-CoV-2 genome. However, they do cover three of the four critical sites distinguishing the D614G/TTTG lineage from the early CCCA lineage (Table 1). Site 3037 is represented by two sequences with a T, site 14408 by four sequences with a T, and site 23403 by one sequence with a G. This "?TTG" configuration is compatible with that of D614G/TTTG lineage but not with the early CCCA lineage represented by the reference sequence (NC_045512). The inescapable conclusion is that the D614G/TTTG lineage has been circulating in northern Italy in 2019, concurrently with or even earlier than the CCCA lineages represented in early SARS-CoV-2 samples in Wuhan.

Three longest fragments (427, 409 and 778 nt, respectively) match perfectly a D614G/TTTG genome (ACCN: OP777407) sampled in Scotland in March 2020 (as well as other D614G/TTTG genomes sampled in Scotland in April and May 2020). There should be little doubt that the sequence fragments listed in Table 1 belong to D614/TTTG strains, although one may still raise the issue of contamination which I will address later.

The last six sequence fragments in Table 1 cannot be individually allocated to either the D614G/TTTG lineage or the CCCA lineage. They are from highly conserved sections in SARS-CoV-2 genomes and matches perfectly to both early D614G/TTTG and CCCA lineages. Similarly, the four SARS-CoV-2 sequence fragments sampled from human sewage in Brazil (MT925972, MT925973, MT925976, MT925977) [12], two on 2019-11-27 and the other two on 2019-12-11, also match perfectly to early D614G/TTTG and CCCA lineages.

The sequence evidence that the early SARS-CoV-2 sequences from northern Italy belong to the D614G/TTTG lineage is consistent with the dating of the MRCA to the summer of 2019 [5][6]. It is also consistent with the twin-beginnings hypothesis on the origin, spread and evolution of SARS-CoV-2 [13].

How would one know if these early Italian D614G/TTTG sequences did not result from contamination? After all, the first seven viral sequences (Table 1) were submitted to GenBank on May 18, 2021 when D614G/TTTG strains have been around for more than a year. The hypothesis of contamination was advocated in particular by those who think it unlikely to have D614G/TTTG sequences in Italy in late 2019. I will not repeat the cautions against contamination taken by

researchers who sequenced those early samples [10]. Instead, I will provide various lines of evidence suggesting that D614G/TTTG strains may well be circulating in Italy much earlier than most of us have thought.

The first two cases of COVID-19 in Italy were confirmed on Jan. 30, 2020. Among 16 fully sequenced SARS-CoV-2 genomes collected in Italy in February 2020, eight are CCCA strains (OP288481, OP288482, OP288483, OP288484, MT509652, MT509660, MT509661, MT509653) which appeared to cause more serious symptoms and could almost all be traced back to China. The other eight are D614G/TTTG strains (MT509664, MT509667, MW530510, MW530511, MT479216, MT483875, MT483881, MT527178), which appeared to cause only mild symptoms. One D614G/TTTG strain (MT527178) was also circulating in Lazio (including Rome) in February 2020. Thus, D614G/TTTG strains were already frequent in Italy in February 2020.

On Feb. 18, 2020, a 51-year-old man from Scotland came to visit Rome via Lombardy, by his private rental car. He subsequently became the first COVID-19 patient in Scotland, confirmed on Mar. 1, 2020 [14][15]. The chronical of the patient [15] was somewhat cryptic. Mar. 1, 2020 was day +3, and the man travelled to Rome via Lombardy on day -9 (i.e., Feb. 18, 2020). Given the average incubation period of about 12 days, he likely was infected in Lombardy on his way to Rome on Feb. 18, 2020. The patient exhibited only mild symptoms throughout admission with no supportive care provided [15]. He developed COVID-19 symptoms on Day 0 and his fever subsided on Day +2. He might not have been diagnosed as the index case of COVID-19 in Scotland if there were no global alert following the viral outbreak in Wuhan.

The SARS-CoV-2 genome from the patient, belonging to the D614G/TTTG lineage, was named Scotland/CVR01/2020 (GISAID: EPI_ISL_413221). Thus, while it remains unclear how D614G/TTTG strains moved into Italy, these viral strains might have been circulating cryptically in Italy for some time, only to be revealed after the import of the more virulent CCCA strains into Italy in late January, 2020. Simultaneous import of both the CCCA strains and the D614G/TTTG strains, although not impossible, should be a low-probability event. Even if such an event did happen, it would still imply that that D614G/TTTG strains have been circulating elsewhere before they were transmitted into Italy. Fig. 1 included a number of D614G/TTTG and TTCG strains reported elsewhere in January and February 2020. Putting all these together, there is really nothing extraordinary to find D614G/TTTG strains in Italy (or anywhere else) in late 2019. The sequence fragments in Table 1 provided more direct confirmation of early existence of D614G/TTTG strains than inferences based on collection dates of genomic records.

These sequence fragments have been overshadowed by the huge number of full-length high-quality SARS-CoV-2 genomes and forgotten for too long [10]. In spite of their high relevance, these sequence fragments (Table 1) have not been used in any previous studies addressing the "When" and "Where" questions, including the recent study by Pekar et al.[16].

Fig. 1 is a phylogenetic tree including early SARS-CoV-2 genomes from Europe and USA, together with the reference genome (NC_045512) representing CCCA genomes and three TTCG genomes from China. There are many CCCA genomes from China, but they are much more similar to each other than to the three TTCG genomes. The tree did not include seven GenBank records with an early collection date (Table 2) because their collection dates could be due to

submission error. I emailed the submitters of these sequences in August and September 2022 for confirmation of the collection date but have never received a reply. The collection dates remain unchanged as of today (2022-11-20). The collection date for the middle five genomes in Table 2 are most likely wrong because they have equivalent sequences in GISAID with the collection year of 2021 instead of 2020.

There were two early but now obsolete reasons for placing the root within the shaded clade (Fig. 1). First, the shaded clade represents the earliest SARS-CoV-2 samples from Wuhan and those that can be traced to China. Second, Huanan Seafood Market is likely where the zoonotic transmission occurred. Associated with these two reasons is the assumption that SARS-CoV-2 was sampled and sequenced right after its presumed zoonotic transmission [17]. The specific placement of the root at the red dot or within the red clade was tenuously based on two nucleotides at sites 8782 and 28144 [17][18]. Lineage A (represented by the red clade in Fig. 1) has T and C at these two sites, and the close relatives of bat-derived viruses such as RaTG13 also have T and C at these two sites. This TC configuration is therefore assumed to be ancestral. In contrast, the CT configuration at these two sites observed in lineage B, which was sampled earlier than lineage A and originally represented by the non-red lineages within the shaded clade (Fig. 1), was deemed derived. Pekar et al.[16] did briefly mention an alternative hypothesis that the TC configuration may not be ancestral because of the existence of SARS-CoV-2 with TT and CC configurations at these two sites indicating an evolutionary trajectory of CT (lineage B)   (TT or CC)   TC (lineage A).

All these interpretations fall apart given the evidence of early circulation of the D614G strain in Table 1. The root of SARS-CoV-2 is more likely at the black dot than at the red dot (Fig. 1). This new root immediately answers two key questions raised by Pekar et al.[16] at beginning of their paper: (i) why were lineage B viruses detected earlier than lineage A viruses and (ii) why did lineage B predominate early in the pandemic? Given a common ancestor indicated by the black dot in Fig. 1, the answer to the first question is that lineage B was detected earlier than lineage A because lineage B appeared originated earlier than lineage A. The answer to the second question is the same, i.e., the ultimately unsuccessful lineage A was not present early in the pandemic (it originated only later) so lineage B predominated early in the pandemic. The answers to these questions would become difficult if the root is at the red dot as assumed by Pekar et al. [16].

There are a number of unresolved problems concerning the origin of the CCCA lineage and the D614G/TTTG lineage. SARS-CoV-2-like coronaviruses isolated from bats and pangolins in Southeast Asia also feature the CCCA signature. What remains controversial is what happened between an ancestral CCCA lineage in the natural coronavirus reservoir and the viral strain causing the Wuhan outbreak. The speculative lab-leak hypothesis states that the ancestral CCCA lineage first entered a lab and then infected human. Two sub-hypotheses exist, one claiming that the lab-leak occurred in a Chinese laboratory and the other claiming that the lab-leak occurred in a US laboratory. The natural origin hypothesis states that the ancestral CCCA lineage infected human through a zoonotic transmission.

The origin of the D614G/TTTG lineage is even less clear. If it is derived from an CCCA lineage, then that CCCA lineage would have to be circulating much earlier than the Wuhan outbreak. Recent dating results do suggest a much earlier common ancestor of SARS-CoV-2 genomes [5][6]. It is even possible, although not highly probable, that the

coronavirus reservoir in nature has two separate lineages (i.e., CCCA and D614G/TTTG lineages) that were transmitted to human independently. At present, the gap is wide between the possible and the actual.

Sequence data currently available is inadequate for one to infer when and where the D614G/TTTG strain originated. Although D614G with the TTTG configuration was not found in China except for imported cases, viral genomes with TTCG configuration were later found in China through retrospective sequencing[19]. What is clear is that viral lineages with the TTTG/TTCG configuration coexisted with the CCCA strain at the time when the latter caused the viral outbreak in Wuhan. Both the TTTG clade and the CCCA clade are likely descendants of a much earlier common ancestor, but the TTTG clade might have descended from one of the CCCA lineages. If scientists around the world would respond to WHO's call to sequence archived samples, then there is a chance to find when and where SARS-CoV-2 originated, either through a natural zoonotic event or a lab-leak event. This task is obviously equivalent to searching a needle in a haystack, given the necessarily low rate of SARS-CoV-2 circulation in the pre-pandemic period. However, there is no alternative for one to get a more precise answer to the "When" and "Where" question. Limiting the search for a common ancestor within the early Wuhan clade is reminiscent of a mink government insisting that the common ancestor of SARS-CoV-2 is within the mink farm in the Netherlands where the first SARS-CoV-2 outbreak was recorded [20].

Acknowledgments

Figures and Tables

Table 1. All SARS-CoV-2 sequences sampled in 2019 in Lombardy and nearby Turin in Italy and deposited in GenBank. The sequence ID is in the form of Accession_Collection date_Location. No sequence includes site 241 but some include sites 3037, 14408 and 23403.

Sequence ID	Start[1]	End[1]	3037[2]	14408[3]	23403[4]
MZ223388_2019-10-22_Lombardy	2977	3185	T		
MZ223391_2019-11-22_Lombardy	2977	3185	T		
MZ223389_2019-10-17_Lombardy	14366	14655		T	
MZ223387_2019-10-19_Lombardy	14366	14655		T	
MZ223386_2019-10-23_Lombardy	14366	14655		T	
MZ223392_2019-12-15_Lombardy	14366	14655		T	
MZ223393_2019-12-15_Lombardy	22904	23681			G
MW303957_2019-12-05_Milan	22935	23343			
MZ223385_2019-09-12_Lombardy	22904	23330			
MZ223390_2019-10-12_Lombardy	22904	23330			
MT843234_2019-12-18_Milan	18484	18770			
MT843235_2019-12-18_Turin	18484	18770			
MT843236_2019-12-18_Turin	18484	18770			

[1] *Start and ending site of the sequence with site numbering according to the reference genome (NC_045512)*

[2] *Synonymous substitution from UUC to UUU codon (both encoding phenylalanine) in protein nsp3 .*

[3] *Nonsynonymous substitution from proline codon CCU to leucine codon CUU in protein RdRp.*

[4] *Nonsynonymous substitution from aspartate (D) codon GAU to glycine (G) codon GGU in protein S.*

Table 2. Controversial early SARS-CoV-2 samples in the D614G lineage with collection date (Coll. Date), country, and Type (nucleotide configuration at sites 241, 3037, 14408 and 23403). Submitters do not respond to request for collection date confirmation.

Accession	Coll. Date	Country	Type
ON085102	2020-01-01	USA	TTTG
MZ047270	2020-01-20	Poland	TTTG
MZ500923	2020-01-07	USA	TTTG
MZ500486	2020-01-20	USA	TTTG
MZ500330	2020-01-21	USA	TTTG
MZ500751	2020-01-25	USA	TTTG
EPI_ISL_8311708	2020-01-10	Lebanon	TTTG
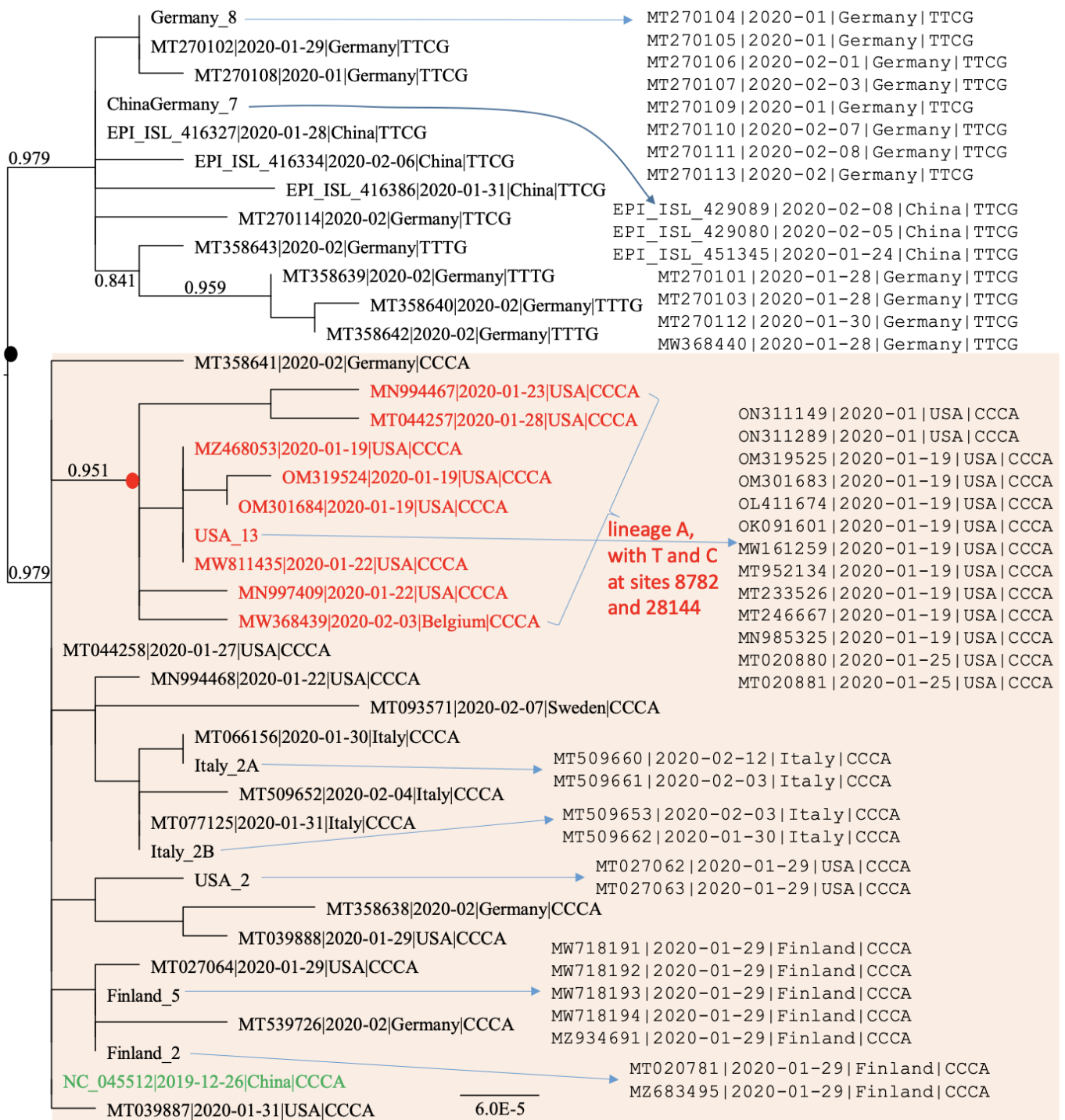
Fig. 1. Phylogenetic tree of early lineages of SARS-CoV-2 isolated in Europe (excluding UK) and USA, together with seven key representative SARS-CoV-2 genomes from China, including the reference genome (NC_045512, colored green). All genomes have at least 29740 nt, and "human" as host. Collection dates were up to February 2020 for European sequences, and by Jan. 31, 2020 for USA sequences. OTU names are in the form of accession|collection date|country|type where "accession" is either GenBank or GISAID accession and "type" is defined by the four nucleotides at sites 241, 3037, 14408 and 23403 that separate the D614G (TTTG) lineage from those early strains isolated in China (CCCA). Viral genomes missing any of these four sites were excluded. Lineage A is characterized by C8782T and T28144C. Identical genomes are represented by a single ID (e.g., Germany_8) but with an arrow pointing to a complete list. The shaded area includes the original viral classification of lineage A and lineage B. Genomes were aligned by MAFFT[21] with the FFT-NS-2 option. The unrooted phylogenetic tree was reconstructed with PhyML[22], with a GTR model and optimization of topology, branch lengths and rates.

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