First Expansion of the Public Tomato Brown Rugose Fruit Virus (ToBRFV) Nextstrain Build; Inclusion of New Genomic and Epidemiological Data

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Abstract

Tomato brown rugose fruit virus (ToBRFV) is a tobamovirus that was first detected in Israel and Jordan following an outbreak of a new disease infecting tomato in 2014. Since then, the virus has been reported from all continents except Oceania and Antarctica. In response to the first finding of the virus in The Netherlands, the Dutch National Plant Protection Organization created a ToBRFV Nextstrain build (v1). In this report, we announce 47 new (near) complete ToBRFV genomes and the generation of the new ToBRFV Nextstrain (v2) build containing 118 ToBRFV genomes with associated geographic and epidemiological data. Examples of utilization of the genomic sequences are presented, and we report the first sequence from South America and present a novel hypothesis on the possible ToBRFV center of origin.

Announcement

Tomato brown rugose fruit virus (ToBRFV) is a tobamovirus that was first detected in Israel and Jordan following an outbreak of a new disease infecting tomato (*Solanum lycopersicum*) in 2014 (Luria et al. 2017; Salem et al. 2016). In addition to tomato, the virus has been reported to infect pepper crops (*Capsicum* spp.) (Cambrón-Crisantos et al. 2019; Panno et al. 2020; Salem et al. 2020) and, under laboratory conditions, several other plant species such as *Petunia* sp. and *S. nigrum* (Luria et al. 2017). Since its first description by Salem and colleagues in 2016, the virus has been reported from all continents except Oceania and Antarctica. In the European Union, ToBRFV is currently regulated as part of EU Commission implementing decision 2020/1191 (Anonymous 2020). Following the first finding of the virus in The Netherlands, an interactive ToBRFV Nextstrain build was created to identify possible links between ToBRFV genotypes, epidemiological traits, and distribution (van de Vossenberg et al. 2020). The ToBRFV Nextstrain build published by van de Vossenberg et al. (2020) is referred to as version 1 (v1). In this report, we announce the release of 47 new (near) complete ToBRFV genome sequences with associated metadata, and the second version of the Nextstrain ToBRFV build, which can be accessed via <https://nextstrain.nrcnvwa.nl/ToBRFV/20201206>.

For the generation, assembly, and annotation of the sequences of (near) complete ToBRFV genomes in this announcement, the methodology described by (van de Vossenberg et al. 2020) was followed. In short, RNA was extracted from fruit, leaves, sepals, stems, or seed of *S. lycopersicum* or *Capsicum* spp. using the Sbeadex Maxi Plant Kit (LCG Genomics) on the automated KingFisher Flex 96 platform (Thermo Fisher). Samples that produced cycle quantification values lower than 23 (fruit, fruit sepals, and leaves) or 25 (seed) with one of the real-time reverse-transcription PCR reactions based on primers and probes described by ISHI-VEG (ISF 2019) were selected for Illumina sequencing. De novo assembled and annotated ToBRFV

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sequences obtained from at least 2 Gb of 150-bp paired-end plant ribosomal RNA depleted RNA sequencing data were submitted in NCBI GenBank under accessions MW314091 to MW314137. The corresponding sequence read archives were deposited in the European Nucleotide Archive (ENA) under accessions ERR5032738 to ERR5032791. An overview of data associated with the newly generated sequences is shown in Table 1.

Genomic ToBRFV sequences from this report; eight public NCBI GenBank sequences of (near) complete ToBRFV genomes released between November 2019 and October 2020—MN013187, MN013188, MN549394, MN549395, MN549396, MN815773 (Beris et al. 2020), MT002973 (Chanda et al. 2020), and MT107885 (Fidan 2020)—and the sequences and meta-data from the first version of the ToBRFV Nextstrain build (van de Vossenberg et al. 2020) were

Table 1. Overview of data associated with (near) complete Tomato brown rugose fruit virus (ToBRFV) genome sequences generated in this announcement^a

Sample	Collection date	Country	Host	Material sampled	Real-time RT-PCR results		Number of reads	ARC (x) ^b	SRA accession	NCBI accession
					CSP1325	CaTa28				
32607982	18 Feb 2019	Egypt	<i>Solanum lycopersicum</i>	Fruit	NA	NA	28,820,766	350,900	ERR5032774	MW314092
33314743	25 Nov 2019	Egypt	<i>S. lycopersicum</i>	Fruit	NA	NA	47,007,964	384,951	ERR5032756	MW314094
36689655	20 Dec 2019	Jordan	<i>S. lycopersicum</i>	Seed	3.7	4.8	24,396,862	460,152	ERR5032740; ERR5032746	MW314107
33314313_B	18 Nov 2019	Egypt	<i>S. lycopersicum</i>	Fruit	NA	NA	42,981,588	821,870	ERR5032757	MW314093
33837271_1	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	8.3	5.8	26,539,030	118,996	ERR5032748	MW314095
33837271_2	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	5.0	4.7	18,241,966	98,161	ERR5032749	MW314096
33837296_1	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	9.1	5.7	19,601,282	180,247	ERR5032750	MW314097
33837296_2	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	8.7	4.7	21,865,848	194,936	ERR5032751	MW314098
34169391_1	29 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Sepals	16.2	16.2	28,116,288	821	ERR5032788	MW314099
34169391_2	29 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Sepals	9.6	8.8	27,282,382	77,202	ERR5032789	MW314100
34169402_1	29 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	11.5	11.4	25,678,314	94,077	ERR5032790	MW314101
34169402_2	29 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	12.2	11.6	30,418,834	123,593	ERR5032791	MW314102
36638057_3	16 Apr 2020	Netherlands	<i>S. lycopersicum</i>	Sepals	15.4	14.8	16,464,630	3,101	ERR5032764; ERR5032781; ERR5032782	MW314103
36689436_1	20 Dec 2019	Jordan	<i>Capsicum annuum</i>	Seed	21.0	21.5	13,729,434	189	ERR5032755	MW314104
36689444_1	29 Jan 2020	Jordan	<i>Capsicum annuum</i>	Seed	11.3	12.1	39,559,582	2,896	ERR5032761	MW314105
36689516_1	9 Mar 2020	Netherlands	<i>S. lycopersicum</i>	Seed	16.2	16.5	20,132,298	636	ERR5032758	MW314106
36689663_1	20 Dec 2019	Jordan	<i>S. lycopersicum</i>	Seed	4.1	4.6	14,981,458	300,206	ERR5032741; ERR5032747	MW314108
36689751_1	20 Dec 2019	Jordan	<i>S. lycopersicum</i>	Seed	13.2	13.2	22,898,576	11,189	ERR5032738	MW314109
36689794_1	20 Dec 2019	Jordan	<i>S. lycopersicum</i>	Seed	2.1	2.1	13,455,782	282,125	ERR5032739	MW314110
36783571_2	31 Mar 2020	Peru	<i>S. lycopersicum</i>	Seed	14.4	13.7	40,560,220	6,832	ERR5032765	MW314111
36783668_2	7 Apr 2020	Netherlands	<i>S. lycopersicum</i>	Seed	20.0	21.4	46,172,096	34	ERR5032766	MW314112
36783756_2	4 Jun 2020	Israel	<i>Capsicum annuum</i>	Seed	12.1	12.5	14,073,884	672	ERR5032767	MW314113
36783860_1	14 Apr 2020	China	<i>S. lycopersicum</i>	Seed	10.0	10.1	22,321,696	27,518	ERR5032760	MW314114
36810152_2	19 Mar 2020	Israel	<i>S. lycopersicum</i>	Seed	16.4	17.3	18,388,928	9,156	ERR5032762	MW314115
36810152_3	19 Mar 2020	Israel	<i>S. lycopersicum</i>	Seed	16.5	17.4	14,611,604	54	ERR5032763	MW314116
36810232_3	20 Mar 2020	Netherlands	<i>S. lycopersicum</i>	Seed	15.9	16.2	22,310,498	23,828	ERR5032759	MW314117
38589922_1	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	8.2	5.3	23,373,016	231,541	ERR5032752	MW314118
38589922_2	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	16.6	17.1	25,839,224	1,795	ERR5032753	MW314119
38589922_3	28 Feb 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	8.8	4.9	21,471,486	154,263	ERR5032754	MW314120
39072896_1	26 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	10.7	10.1	24,892,422	108,910	ERR5032744	MW314121
39072896_2	26 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	13.1	12.3	13,127,192	26,779	ERR5032745	MW314122
39563433_3	18 May 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	13.5	13.3	15,531,416	452,193	ERR5032768; ERR5032783; ERR5032784	MW314123
39774038_1	28 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	9.1	5.2	26,196,904	161,215	ERR5032786	MW314124
39774038_2	28 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	5.5	4.1	25,828,516	330,753	ERR5032787	MW314125
39971816_1	25 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	15.1	16.2	26,630,360	1,167	ERR5032742	MW314126
39971816_2	25 Nov 2019	Netherlands	<i>S. lycopersicum</i>	Leaves	18.5	20.1	26,860,324	61	ERR5032743	MW314127
39985935_4	22 Jun 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	9.5	7.4	89,389,096	1,535,600	ERR5032769; ERR5032785	MW314128
40001980_D	8 Oct 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	11.0	10.3	18,267,336	62,319	ERR5032780	MW314129
40002000_A	30 Jul 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	12.2	12.2	16,539,462	75,403	ERR5032773	MW314130
40002166_A	30 Jun 2020	Netherlands	<i>S. lycopersicum</i>	Sepals	3.6	2.7	70,199,120	1,123,963	ERR5032770	MW314131
40002350_A	5 Aug 2020	Netherlands	<i>S. lycopersicum</i>	Sepals	4.0	5.4	24,866,542	377,463	ERR5032772	MW314132
40002377_1A	3 Sep 2020	Netherlands	<i>S. lycopersicum</i>	Sepals	5.4	4.0	19,760,698	243,797	ERR5032775	MW314133
40002377_2B	3 Sep 2020	Netherlands	<i>S. lycopersicum</i>	Sepals	5.9	4.1	19,395,176	299,016	ERR5032776	MW314134
40002377_3C	3 Sep 2020	Netherlands	<i>S. lycopersicum</i>	Sepals	3.1	2.9	26,646,018	451,723	ERR5032777	MW314135
40002385_A	15 Sep 2020	Netherlands	<i>S. lycopersicum</i>	Leaves	4.3	4.7	21,505,372	450,466	ERR5032778	MW314136
40002393_C	5 Aug 2020	Netherlands	<i>S. lycopersicum</i>	Stems and leaves	16.9	16.8	32,230,150	464	ERR5032771	MW314137
6189975_2	19 Oct 2020	China	<i>S. lycopersicum</i>	Seed	18.0	19.8	27,721,922	4,034	ERR5032779	MW314091

^a SRA = Sequence Read Archive. NA = not available; sample was included for Illumina sequencing based on other methods such as enzyme-linked immunosorbent assay, indicator plants, or other real-time reverse-transcription PCR (RT-PCR) tests.

^b Average read coverage (ARC) of the (near) complete ToBRFV genome.

used to create the ToBRFV Nextstrain (v2) build. The new build was generated as described by van de Vossen et al. (2020), except for the Augur Export command version (i.e., v2 instead of v1), allowing customization of the webpage. The ToBRFV Nextstrain (v2) build contains 118 genomic sequences from 15 countries from materials sampled between November 2014 and October 2020 (Fig. 1A). New in the ToBRFV Nextstrain (v2) build is information on the country of origin of rootstock and scion seed. In addition, major clades have been defined to facilitate the communication of genomic ToBRFV diversity. The augur input data and scripts are available on GitHub via <https://github.com/NPPO-NL/nextstrain-ToBRFV>.

The new sequence data and the associated metadata as presented in the ToBRFV Nextstrain (v2) build are instrumental for the plant virological research community to better understand the diversity and epidemiology of ToBRFV in global context. For regulatory bodies, such data can be helpful to take risk-based measures and identify or disqualify potential outbreak sources. An example of the latter is to determine the effect of cleaning and disinfection of affected greenhouses during crop rotation. Dutch fruit companies that were found to have ToBRFV at their premises intensively cleaned and disinfected their facilities during crop rotation. Unfortunately, in some cases, ToBRFV was detected again at these locations 3 to 6 months into the new ToBRFV host production cycle. Sequences generated from those new infections could be used to determine whether companies had not managed to eliminate the virus from their facilities or reintroduced the virus from the same source, or if those companies contracted the virus from another source. Examples of both cases are represented in the ToBRFV Nextstrain (v2) build (Fig. 1B and C). In one case, two identical sequences from clade 6 were obtained following sampling in October 2019 (MN882042 and MN882043) at a production

Fig. 1. Tomato brown rugose fruit virus (ToBRFV) Nextstrain v2. **A**, Genomic epidemiology of 118 (near) complete ToBRFV genomes sampled between October 2014 and October 2020 represented as a time tree (left panel) and the geographic distribution (right panel). Examples of tomato production locations where ToBRFV was found in 2019 and (after intensive cleaning and crop rotation) again in 2020 are presented. **B**, In one case, a nearly identical sequence was found in the year following the initial infestation and **C**, in another case, sequences from a different cluster were identified in the year following the initial infestation.

location in the Dutch municipality of Brielle. After cleaning and crop rotation, ToBRFV was found again in the new tomato crop several months later, producing a sequence nearly identical (MW314123: 3 single-nucleotide polymorphism [SNPs]; 99.95% sequence similarity) to the previously sequenced material. In this case, it is likely that the company did not manage to fully eliminate the virus from their facilities. In another case, ToBRFV was detected in November 2019 at a production location in the Dutch municipality of Reimerswaal. Sequencing of the obtained material resulted in two identical clade 1 sequences with (MN882059 and MN882060). In September 2020, ToBRFV was detected again at the same production site and, again, three identical sequences were obtained (MW314133, MW314134, and MW314135). However, the 2020 sequences form a unique cluster and differed by 22 SNPs (= 99.65% sequence similarity) compared with the 2019 sequences. For this production site, it is likely that the virus was introduced anew from another source.

Among the newly generated sequences is one (near) complete ToBRFV genome obtained from tomato seed from Peru (MW314111). This sequence represents the first sequence from a South American country, and is (together with the sequences found at one Dutch outbreak location) early diverging in the RAXML time tree. This early-diverging lineage suggests that more sequence diversity can be expected and, currently, not all intraspecific variation is known. Given the position of the Peruvian sequence in the tree, one could speculate that the center of origin of ToBRFV might not lie in the region from where it was first described (i.e., the Middle East) but could lie in South America. Phylogenetic analyses from tobamoviruses and their solanaceous crops were shown to be congruent (Gibbs et al. 2015), which supports our hypothesis that ToBRFV might originate from the presumed center of origin for the Solanaceae family (i.e., South America) (Olmstead 2013). Because the sequence diversity is expected to be highest at the center of origin, those sequences are expected to be broadly represented in the tree, particularly in the early-diverging branches. With 82 of 118 sequences originating from materials sampled at outbreak locations in The Netherlands, there is a strong sampling bias toward Dutch ToBRFV outbreak locations. To reliably test the South American center-of-origin hypothesis, more sequences from South America and other locations worldwide are needed.

The predictive power of the ToBRFV Nextstrain build will improve with the inclusion of additional genomes and metadata. Therefore, we encourage other organizations to share sequence data or biological materials together with relevant metadata to improve the build and our understanding of the diversity and spread of this important virus.

Data Availability

The (near) complete ToBRFV genomes assembled and annotated in this study were submitted to NCBI GenBank under accessions MW314091 to MW314137. The corresponding sequence read archives were deposited in ENA under accessions ERR5032738 to ERR5032791. The ToBRFV Nextstrain (v2) build, containing 118 ToBRFV genomes with associated geographic and epidemiological data, is available via <https://nextstrain.nrcnvwa.nl/ToBRFV-internal/20201206>, and the underlying Augur input data and scripts are available via the National Plant Protection Organization of The Netherlands (NPPO-NL) GitHub repository via <https://github.com/NPPO-NL/nextstrain-ToBRFV>.

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