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Short Notes

Symptomatic, widespread, and inconspicuous: new detection of tomato fruit blotch virus

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Summary. Tomato production is an important part of the Swiss vegetable production with most tomato crops grown in greenhouses. Tomato plants are vulnerable to diseases caused by viruses, which can have significant impacts on crop production. This study reports the first detection of tomato fruit botch virus (ToFBV, *Blunervirus solani*) in Switzerland, from a tomato production site at the southern part of the Ticino region. The symptoms observed indicated presence of a viral pathogen, but tests against the most common tomato viruses were negative. Immunocapture of double-stranded RNA and its subsequent sequencing on a Flongle flowcell (Oxford Nanopore Technologies) identified the presence of ToFBV and southern tomato virus. The genome of the Swiss ToFBV isolate was very similar to that available in GenBank. Datamining of the sequence read archives found the virus in two other countries, with a highly conserved genome. With this study, there are now 12 near-complete genomes of ToFBV available, and the virus is recorded from ten countries. This study underlines the importance of continuous monitoring and research on emerging viruses in tomato production.

Keywords. *Kitaviridae, Blunervirus solani*, Flongle sequencing, dsRNA.

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INTRODUCTION:

Tomato production (*Solanum lycopersicum*) is an important part of the Swiss agricultural sector, with more than 40,000 metric tons produced in 2021. Of the production area, 4% was in open fields, and 96% was in greenhouses, with almost 60% using the soilless production systems (Swiss Federal Statistical Office, 2022). Tomato plants are vulnerable to a range of pests and diseases, often depending on climate, geographical location, and production system. These pests and pathogens include insects, nematodes, fungi, oomycetes, bacteria, and viruses (Panno *et al.*, 2021). A significant number of pathogens are well-known, researched, and controlled; however, the industry is also confronted with emerging diseases, many of which are associated

with viruses. In a recent review, Rivarez *et al.* (2021) listed more than 312 virus, satellite virus, satellite RNA or viroid species (in 22 families and 39 genera) associated with tomato.

In the last decade, tomato brown rugose fruit virus (ToBRFV) has rapidly spread across 35 countries, and has had significant impacts on world tomato production (Caruso *et al.,* 2022). ToBRFV was reported in Switzerland in 2021, from soil-grown tomato production in the north-east of the country (Mahillon *et al.,* 2022). Another, lesser-known symptomatic and emerging virus, tomato fruit blotch virus (ToFBV, *Blunervirus solani*), was identified in 2020 in Italy and Australia (Ciuffo *et al.,* 2020). ToFBV is in the family *Kitaviridae*, a group of plant viruses distantly related to nege-like viruses that mainly infect invertebrates (Ramos-González *et al.,* 2022). In the past 2 years, the virus was detected in Spain, Portugal, Brazil, Tunisia and Slovenia (Kitajima *et al.,* 2022; Maachi *et al.,* 2021; Nakasu *et al.,* 2022; Rivarez *et al.,* 2022). The virus was detected from symptomatic tomato plants, and uneven and/or deformed fruits were indicated when the symptoms were precisely described (Ciuffo *et al.,* 2020; Kitajima *et al.,* 2022; Nakasu *et al.,* 2022).

The present study reports the first detection of ToFBV in Switzerland, and extends the probable distribution of this virus through mining of publicly available sequence read archive (SRA) data.

MATERIAL AND METHODS

In August 2022, an inspector from the Ticino Agricultural Advisory Office visited a tomato production site in the south of the Ticino region (the southern tip of Switzerland) in response to a request from the producer. Tomato plants were grown in soil under plastic tunnels. Because of the unusual nature of the symptoms observed, samples were collected for laboratory analysis. Severe symptoms observed on fruits included chlorotic rings and sometimes distortion, and these indicated presence of a viral pathogen (Figure 1). After confirming the absence of tomato brown rugose fruit virus (ToBRFV) using specific RT-qPCR, samples were tested for 16 additional known virus, viroid or phytoplasma pathogens of tomato, using lateral flow devices, ELISA, RT-PCR and RT-qPCR. All of these tests were negative, and no particles were observed using a transmission electron microscope.

A double-stranded RNA (dsRNA) extraction was prepared by immunocapture (Blouin *et al.*, 2016). Approximately 3 g of frozen leaf tissue were ground in liquid nitrogen and added to 11.2 mL of extraction buffer (STE with 0.3% bentonite, 2% PVP, 1.5% SDS and 2% beta-mercaptoethanol). The extract was then added to 8 mL of phenol in a 50 mL tube. The sample was vortexed for 1 min, and then centrifuged for 4 min at 2,200 *g*. From the aqueous phase, 6.4 mL was collected and added to 5.1 mL of isopropanol in a new tube. The sample was kept on ice for 10 min, and total nucleic acids were then precipitated by centrifugation at 17,000 *g* for 20 min at 4°C. The resulting pellet was rinsed twice with 75% ethanol, air dried for 10 min, and then resuspended in 2 mL Tris-buffered saline-tween (TBST, 25 mM Tris, 0.15M NaCl, pH 7.5 + 0.05% Tween). From the 2 mL, 1.5 mL were used for the immunocapture, and the remaining 0.5 mL was then kept in a -20°C freezer. Single-stranded RNA was digested with 187.5 U of RNAse T1 (Thermo Fisher Scientific) for 1 h at 37°C on a thermal mixer.

Immunocapture of the dsRNA was prepared as described by Blouin *et al.* (2016). A total of 10 µL of Protein L magnetic beads (Thermo Fisher Scientific) was washed three times in TBST buffered as per the manufacturer recommendation. A total of 400 µL of monoclonal antibody hybridoma supernatant 2G4 (O'Brien *et al.,* 2015; UniQuest Pty Limited) was added to the beads, and these were then incubated at room temperature for 1 h on a rotary mixer. The beads were then washed three times in TBST, resuspended in TBST and added to the RNAse T1 digested extract. The sample was then incubated 1 h at room temperature on a rotary mixer. The beads were then washed three times with TBST buffer, air dried for a few minutes, and resuspended in 20 µL of ultrapure water. The cDNA was synthetized from the dsRNA after an initial heating at 99°C for 2 min of 9 µL of the resuspended beads with 4 µL of linker primer CGTGGAGACTCTGGNNNNNNNNNNT at 1 µM. The sample was then immediately placed on ice, and the mix was completed with dNTPs (0.5 mM final), 4 µL of 5x Buffer RT, 1 µL of ultrapure water, 20 U of RNAsin® Ribonuclease inhibitor (Promega Corporation), and 100 U of Maxima H Minus (Thermo Fisher Scientific). The sample was kept on ice for 15 min, then incubated for 10 min at 25°C, followed by 30 min at 50°C, and the enzymes were deactivated with a final step of 5 min at 85°C. Remaining RNA was removed by adding 0.75 µL of RNAse A (20 mg mL^{-1}) and incubated for 15 min at 22°C and 2 min at 85°C.

The cDNA was then purified with the AMPure XP (Beckman Coulter) following the manufacturer recommendations, and eluted in 30 µL. PCR was then carried out with the LongAmp Taq 2× Master Mix (New England Biolabs), with 5 µL cDNA, 5 µL MID primer (multiplex identifier) AAGGTAGAAGCGTGGAGA-

CTCTGG, and 10 µL of mastermix. The initial cycle was 95°C for 5 min, 65°C for 30 min and 75°C for 1 min, then followed by 30 cycles each of 94°C for 30 sec, 50°C for 30 sec and 72°C for 3 min, and a final extension of 10 min at 72°C. The sample was then loaded on an agarose gel to visualize the band size. The PCR was cleaned with the AMPure XP (Beckman Coulter) following the manufacturer recommendations and eluted in 20 µL. A DNA concentration of 55 ng μL^{-1} was measured by Qubit Fluorometric Quantification (Thermo Fisher Scientific), and the median size of the DNA amplicon was estimated to be 1,200 bp from the agarose gel. A total of 0.65 µL of the cleaned PCR product was used in the ligation to a concentration of 50 fmol. The ligation sequencing amplicons (kit SQK-LSK110, Oxford Nanopore Technologies) was used as recommended by the manufacturer. The sample was loaded on a Flongle (68 active pores at start) for a 24 h run, and sequenced alongside with another extract from a different plant (*Vitis vinifera* L.), as part of a different experiment but following the same protocol with a different linker and MID.

Virus sequences were recovered from the data by mapping against a reference database of plant viruses using Minimap2 (2.24) plugin (Li, 2021) on Geneious Prime (v2022.0.2 https://www.geneious.com/). The presence of ToFBV was confirmed by RT-PCR using the primers of Nakasu *et al.* (2022), and a gap in the RNA3 was filled with the following primers; ToFBV-RNA3_1823 F (TCTTCGGTCTGCTCGTGATG) and ToFBV-RNA3_2777 R (CGAAACAGAGACCCGTCCAA). Amplicons were Sanger sequenced. Genome reconstruction was carried out using Geneious Prime.

Datamining was carried out to find additional evidence of the virus, using Serratus (https://serratus.io/) to screen publicly available SRAs deposited before January 2021 (Edgar *et al.,* 2022). The positive SRA files were imported in the Galaxy platform (usegalaxy.org, The Galaxy Community, 2022), where the genomes of ToFBV were reconstructed using a combination of *de novo* sequencing with rnaviralSPAdes (Galaxy Version 3.15.4; Prjibelski *et al.*, 2014; Antipov *et al.*, 2015; Vasilinetc *et al.*, 2015) and reference mapping (Bowtie2 version 2.5.0; Langmead *et al.,* 2009).

RESULTS AND DISCUSSION

From the Flongle sequencing run, a total of 160,241 reads were recovered (167 Mb) with a N50 of 895 nt. Five viruses and one viroid were retrieved from the grapevine sample. Two non-grapevine viruses were detected from the tomato sample, and these were : southern tomato virus (*Amalgavirus lycopersici*) with 18,835 mapped reads, 100% horizontal coverage and 99.97% similarity to isolate Thailand LC487710; and ToFBV, where a total of 38,090 reads mapped the four RNAs with horizontal coverage greater than 98% (Table 1).

Southern tomato virus is a seed-borne virus that is most often asymptomatic. It is widespread, as shown by the 129 accessions deposited in GenBank to date from 25 countries, including Switzerland (Sabanadzovic *et al.,* 2009; Turco *et al.,* 2018).

The four almost complete polyadenylated segments of ToFBV shared the same structure as the other members of the species. All segments recovered were contiguous (only the extremities missing), with the exception of the RNA 3 where a short gap was observed near the 3' end. This gap was completed by RT-PCR. The largest RNA fragment (5,606 nt) encodes a large polyprotein with a methyl-transferase and a helicase recognized domains, the second RNA (3580 nt) encodes a polyprotein with a viral helicase and the RNA-dependent RNA Polymerase (RdRP) domains. The RNA 3 (2755 nt) contains five putative ORFs including one coding the SP24 superfamily motif (putative virion membrane proteins). The RNA 4 (1924 nts) contains two putative ORFs including one coding a putative movement protein.

The four RNA segments recovered were deposited in GenBank (OQ849577- OQ849580). Blast analyses confirmed the close relationship among the virus isolates available. The four Swiss RNA segments closest matches

Table 1. Molecular features of the four RNA segments of tomato fruit blotch virus detected in Switzerland and closest isolate.

RNA	Length (nt)	Horizontal coverage (%)	Read mapped	Nucleotide identity by nBlast[a] (%)	Closest accession by nBlast (Country)
RNA1 (OQ849577)	5,606	99.3	521	99.42	MZ401001 (Tunisia)
RNA2 (OQ849578)	3,580	99.3	221	99.36	MW546268.1 (Brazil)
RNA3 (OQ849579)	2,755	99.1	10,799	98.44	OL472085.1 (Slovenia)
RNA4 (OQ849580)	1,924	98.4	26,549	99.53	MK517480.2 (Italia)

[a] Query coverage >98% and e-value = 0.

Figure 1. Tomato fruit symptoms observed from tomato fruit blotch virus infested site in Ticino, Switzerland.

were from four different isolates, with the percentage identity greater than 98% (Table 1).

ToFBV was also detected by datamining using Serratus (https://serratus.io/). The palmID analysis of viral RdRP identified palmprint with 100% homology to ToFBV from two different bioprojects: one SRA from the bioproject PRJNA626066 "Metaviromic analysis of South Africa sweet potato" (SRR11566106), and three SRAs from the PRJNA491201 "Tomato fruit inoculated with various fungal pathogens" (SRR7841169; SRR7841291; SRR7841300 tomato inoculated with *Fusarium acuminatum* or *Rhizopus stolonifer*) (Petrasch *et al.,* 2019). Nucleotide sequence data reported are available in the Third-Party Annotation Section of the DDBJ/ENA/GenBank databases, under the accession numbers TPA: BK063407 to BK063422. All the genomes showed high degrees of homology, with >95% nucleic acids identity on the four ToFBV RNA segments.

With the addition of the Swiss isolate and the four isolates obtained from the SRAs, there are now 12 near-complete genomes of ToFBV available. Although all publications of ToFBV to date have reported tomato as the only host, GenBank accessions of the Tunisian isolate indicate that the virus was sequenced from potato (*Solanum tuberosum* L.). Similarly, the South African isolate was reconstructed from SRA originating from sweet potato (*Ipomoea batatas*). These two non-tomato hosts should be confirmed by a complementary assay to validate the new host-virus associations. Nevertheless, it is notable that this virus was first described 3 years ago, but is now present, with a highly conserved genome, in ten countries across five continents (Australasia, Eurasia, Africa, North and South America)

This wide distribution resembles that observed for Physostegia chlorotic mottle virus (PhCMoV, *Alphanucleorhabdovirus physostegiae*) first reported in 2018 and then rapidly reported in several countries (Temple *et al.,* 2022). As with ToFBV, most of the PhCMoV sequences in GenBank are almost complete genomes. As diagnostic tools are not yet available, high-throughput sequencing (HTS) is being used to determine the unidentified viral pathogens. PhCMoV also has a highly conserved genome, and the symptoms on the tomato fruits could be mistaken. However, the occurrence of PhCMoV has been, to date, restricted to Europe (Temple *et al.,* 2022).

As observed with PhCMoV, ToFBV can remain in the environment undetected for some time, as suggested by the 10-year-old isolates sequenced from Italy (Ciuffo *et al.,* 2020) and Tunisia. The rarity and sporadic detection of ToFBV contrasts with its worldwide distribution and its conserved genome, although this has also been observed with some other members of the *Kitaviridae* (e.g., *Cileviruses*). The improved surveillance and detection, for example with the rise of HTS technology to detect plant viruses, can partially explain the widespread detections, although this technology has been applied in many laboratories for most of the last decade. Global trade could also explain how the virus and its suspected mite vector are transported across continents (Ramos-González *et al*., 2023). The conserved genome of the virus is also probably the result of better and longer adaptation to its vector than to its host plants. Kitavirids arise from interkingdom virus transfer (Doljan *et al*., 2020). They are the only plant viruses in their phylum with enveloped virions, and they have molecular and biological characteristics likely inherited from an ancestor shared with the arthropod nege-like viruses. Kitavirids may be well-adapted to their mite vectors, whose fitness can be enhanced upon infection compared to their plant hosts, where they lack long-distance movement capability resulting in non-systemic infection (Ramos-González *et al*., 2023).

The present study reports the first detection of ToFBV in Switzerland. Reports of the virus from Italy and Brazil showed its recurrence on the infested sites (Ciuffo *et al.,* 2020; Kitajima *et al.,* 2022). The site where the Swiss isolate was identified will be monitored to increased knowledge of the epidemiology of the virus.

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