Survey of aphid-transmitted viruses and associated RNA molecules in Swedish sugar beet crops

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Abstract

Sugar beet is a crop of great economic importance, contributing significantly to global sugar production. Several viruses that aphids spread across the sugar beet crop have been managed with the application of neonicotinoid insecticides. The Scandinavian sugar beet industry has seen a rise in the number of diseases transmitted by aphids due to the prohibition of the use of neonicotinoids in 2018. The aphid-transmitted disease "virus yellows" has emerged as a major threat to Sweden's sugar beet crop in recent years. As of now, three poleroviruses, namely beet mild yellowing virus (BMYV), beet chlorosis virus (BChV) and turnip yellows virus (YuYV), have been identified in Sweden as associated with the disease. Beet yellows virus has also been identified in Sweden and can cause virus yellows.

The objectives of this thesis were to study the diversity of aphid–transmitted viruses that infect sugar beet in the southern region of Sweden and to test for presence of turnip yellows virus-associated RNA (TuYVaRNA) in sugar beet. Symptomatic sugar beet leaves were collected from seven locations in Skåne in 2023. The samples were subjected to a double antibody sandwich enzyme-linked immunosorbent assay showing that samples collected from four locations were infected with poleroviruses. In this thesis, RT-PCR with universal primers for the coat protein gene of poleroviruses confirmed infection with polerovirus in these four positive samples as well as in one negative sample. Sequencing of cloned RT-PCR products revealed that three samples were infected by BChV and two samples by BMYV. In a phylogenetic analysis, one BMYV isolate showed a close relationship to a previously sequenced BMYV isolate from Sweden, while the other BMYV isolate represented a new genotype. The three BChV isolates were different from the previously sequenced Swedish BChV isolates and were most closely related to an isolate from France. The results showed that the diversity of BMYV and BChV in Sweden is higher than previously thought. New virus genotypes can be transmitted by migrating aphids from one country to another. Mixed infection of viruses could lead to emerging new virus variants as well. Using newly designed primers, RT-PCR amplification was carried out for detection of TuYVaRNA, but the tests were negative suggesting that the the tested plants might not have been infected with TuYVaRNA.

Keywords: aphids, beet chlorosis virus, beet mild yellow virus, sugar beet, virus yellows

Popular science summary

Effective disease management is dependent on accurate disease diagnosis. It might be challenging to diagnose the right cause based just on the visible symptoms because various abiotic factors including water stress, drought, and nutrient deficiency can all create similar symptoms. The primary sign of the disease virus yellows, which is caused by several viruses, is the yellowing of sugar beet leaves. Gradually, the disease may spread within the entire field and cause a massive yield loss. According to current research, three main viruses are responsible for viral yellows in sugar beet in Sweden: beet yellows virus, beet mild yellowing virus and beet chlorosis virus. All these viruses are transmitted by aphids. Earlier, neonicotinoid insecticide was used to successfully suppress virus yellows. However, because of the neonicotinoids' severe environmental effects, they have been prohibited in Europe since 2018. Consequently, there is an increased risk for virus yellows disease in Sweden's sugar beet crop.

The current study was able to identify beet mild yellowing virus and beet chlorosis virus in sugar beet field samples that had been collected from five locations in Skåne, 2023. Symptomatic sugar beet leaves collected from two locations were infected with beet mild yellowing virus and leaves from the other three locations were infected with beet chlorosis virus. Analyses showed that the virus isolates identified in the present study are different from those previously found in Sweden, which means that the viruses are more variable than we knew. These new virus variants could have been brought into Sweden by migrating aphids. Because of limited knowledge of the virus, there is a lot of room for new investigations. Breeders developing new sugar beet cultivars to be grown in Sweden can utilize this information to determine the spectrum of resistance that the plants require. It is critical to have thorough knowledge about these viruses to manage infection and stop their spread, hence reducing yield loss due to viral yellows.

Recently it was found that there are some other smaller virus-like molecules living together with those viruses mentioned above called “associated RNA molecules”. Infection of both viruses and associated molecules together can lead to severe yield loss and control strategies would be more challenging than those in single virus infection. However, in this study, associated RNA molecules could not be found in the virus-infected samples.

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Abbreviations

AMV	alfalfa mosaic virus
aRNA	associated RNA
BChV	beet chlorosis virus
BCTV	beet curly top virus
BLAST	Basic local alignment tool
BLCV	beet leaf curl virus
BMYV	beet mild yellowing virus
BNYVV	beet necrotic yellow vein virus
BSBMV	beet soil-borne mosaic virus
BtMV	beet mosaic virus
BWYV	beet western yellows virus
BYNV	beet yellow net virus
BYV	beet yellows virus
cDNA	complementary DNA
CMV	cucumber mosaic virus
CP	coat protein
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay
EU	European Union
H	Hilleshög
L	Lagestorp
M	Maglarp
T	Tågarp
TAE	Tris-acetate-EDTA buffer
tlaRNA	tombusvirus-like associated RNAs
TuMV	turnip mosaic virus
TuYV	turnip yellows virus
TuYVaRNA	turnip yellows virus-associated RNA
V	Vadensjö

1 Introduction

1.1 Sugar beet

Sugar beet (*Beta vulgaris*) is an important crop plant that belongs to the family Amaranthaceae. The primary reason for sugar beet cultivation is the production of white sugar. The roots of sugar beet plants have a high sucrose content and while sugarcane (*Saccharum officinarum*) has a sugar level of 8–10%, sugar beet has a sugar level of 16% (Tayyab et al., 2023). Moreover, sugar beet is utilized in the manufacturing of products such as molasses, pectin, ethanol, bioethanol, pulp, and animal feed. Sugar beet is a short-duration crop (6 months), which requires a low water amount to grow and it is salt-resistant. The low-cost input for this crop makes it increasingly popular all around the world (Garcia Gonzalez and Björnsson, 2022). Sugar beet originates from wild sea beet and there are several other types of cultivated beets such as chard, red beet and fodder beet (Grimmer et al., 2008). Although sugar beet originated in temperate regions, the cultivation of sugar beet has now expanded to sub-tropical and tropical areas (Misra and Shrivastava, 2022).

1.1.1 Sugar beet and viral disease

During every developmental stage, sugar beet crop can be infected with different pathogens in both the root and the shoot. Sugar beet virus infections affect producers all over the world and pose serious risks to crop quality and output. These viruses are mainly spread by soilborne vectors like the protozoa *Polymyxa beta* or by insect vectors like aphids and leafhoppers (Esh and Taghian, 2022).

Numerous viruses of sugar beet are transmitted by insect vectors, including beet curly top virus (BCTV), beet leaf curl virus (BLCV), beet mild yellowing virus (BMYV), beet western yellows virus (BWYV), beet mosaic virus (BtMV), beet yellow net virus (BYNV), and beet yellows virus (BYV) (Esh and Taghian, 2022). Several aphid-transmitted viruses, including BYV, BMYV, beet chlorosis virus (BChV), and BWYV are the cause of the disease virus yellows (VY) (Mahillon et al., 2022).

The root viral disease rhizomania of sugar beet is thought to be the most devastating disease to affect sugar beet globally (EFSA Panel on Plant Health et al., 2020). It is caused by beet

necrotic yellow vein virus (BNYVV) and the obligate, fungal-like parasite *P. betae* is the vector of BNYVV.

1.2. Aphid-transmitted viruses in sugar beet

Hemipteran insects are vectors for a majority of the plant-infecting viruses (Jayasinghe et al., 2022). Notable aphid species that may transmit over 20 different plant viruses include green peach aphid (*Myzus persicae*), the potato aphid (*Macrosiphum euphorbiae*), the ground nut aphid or cowpea aphid (*Aphis craccivora*), and cotton aphid or melon aphid (*Aphis gossypii*) (Jayasinghe et al., 2022). Aphids can transmit viruses in a persistent, semi-persistent or non-persistent manner. To manage virus infection of crops, it is necessary to develop virus-resistant or vector-resistant cultivars, control virus vectors by biological control of insecticides, and adapt cultural practices. Using chemicals on a large scale has negative environmental effects and has been the reason for developing regulations against their usage.

The neonicotinoid insecticide was used successfully for a long period to control the aphid-transmitted viruses of sugar beet. Neonicotinoids were used for sugar beet seed coating (Hauer et al., 2017) with effects against several insect-transmitted viral diseases including those transmitted by whiteflies, thrips or aphids (Laurent et al., 2023). The neonicotinoids have a broad range of effectiveness, protect plants systemically (Francis et al., 2022), are inexpensive, have long-lasting effects, and have many applications and uses, which accounted for their frequent use (Elbert et al., 2008). Nonetheless, due to concerns about the potential negative impact of neonicotinoids on the environment, notably on bees and other non-target animals, their use has been prohibited in the EU since 2018 (European Food Safety Authority, 2018) and the management of several aphid-transmitted viral diseases has become problematic (Romanowski and Blake, 2023).

Poleroviruses are important plant pathogens that are transmitted by aphids and restricted to the vascular tissue of the host plant (Ali et al., 2014). They cause serious damage around the world to important field crops including maize, potato, sugarcane, melon, sugar beet, oilseed rape, wheat, and barley (Gray and Gildow, 2003). They have a single-stranded (+) RNA genome of 5 – 6 kb. The genome contains 7 highly overlapping ORFs and 3 non-coding regions (LaTourrette et al., 2021). The non-coding regions contain important regulatory sequences and those regions also act as hot spots for intra- or inter-species recombination.

One of the most significant diseases caused by polerovirus impacting sugar beet crops is virus yellows (VY) which is transmitted by green peach aphids and black bean aphids (Hossain and

Varrelmann, 2021). Of the four viruses causing VY, one (BYV) belongs to genus *Closterovirus*, family *Closteroviridae*, and the other three (BMYV, BChV and BWYV) belong to genus *Polerovirus*, family *Solemoviridae* (Stevens et al., 2005a). In the plants affected by VY, the lamina between the leaf veins first gradually turns yellow (Figure 1). In June, symptomatic plants appear as round yellow spots in the fields (Esh and Taghian, 2022). After that, the leaves become brittle and thicker. In July and August, symptomatic plants appear as small, localized patches in the fields that can quickly spread to the entire field (Hossain et al., 2021). Reduced photosynthetic area of the leaves, which affects production and sugar content in the beets, can result in up to 50% yield losses (Mahillon et al., 2022). The ban on neonicotinoids has now caused the VY issue to return. As one of the major crops that contribute to the world's sugar supply faces a serious threat to its production, VY has drawn the attention of researchers worldwide. Therefore, numerous research studies have been conducted on VY worldwide, and interesting results from some studies are included here.

Ten countries were included in a study, which showed that the distribution of yellowing viruses varied with latitude throughout Europe, with BYV being more prevalent in the Mediterranean region and BMYV prevailing in the northern and western areas (Stevens et al., 2005b). Moreover, they discovered that in the UK, BChV accounted for 11% of isolates obtained from yellow sugar beet plants in 1990 while by 2003, BChV accounted for more than 25% of the polerovirus isolates. There was a noteworthy increase in BChV detection in sugar beet in the UK according to this study. A more recent investigation conducted in Europe from 2017 to 2019 on viruses causing VY, revealed that there is a natural fluctuation of poleroviruses in the studied countries (Hossain et al., 2021). In Switzerland, VY reappeared for the 2020 season (Mahillon et al., 2022), and it was likely one of the two main causes of the sugar beet production decline that year. These studies show that the level of VY in sugar beet fields has changed in Europe from low to high. Aphid migration may be exacerbated by climatic factors such as warm and dry weather. This helps the virus spread since these vectors may be carried by the wind and scattered across wider areas (Hossain et al., 2021). The growth of the aphid population is greatly aided by the weather conditions of the previous winter and early spring (Bell et al., 2015). Since these viruses may spread over great distances via aphids and have a variety of other hosts, VY will probably

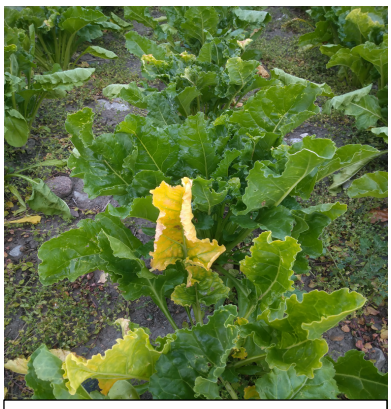

Figure 1: Sugar beet plant in Alnarp, Skåne county, with symptoms of virus yellows.

remain a significant concern over the whole continent. This indicates that global climate change is impacting agriculture both directly and indirectly.

One recent study utilizing seven symptomatic sugar beet leaf samples from the southern region of Sweden revealed that infection with turnip yellows virus (TuYV, genus *Polerovirus*) occurred together with BMYV and BChV (Puthanveed et al., 2023). This was the first identification of BChV and TuYV in sugar beet in Sweden. The discovery of TuYV in sugar beet indicates possible transmission between different host plants (Puthanveed et al., 2023). TuYV has a wide host range including plants of the families Brassicaceae, Chenopodiaceae, Asteraceae and Amaranthaceae (Stevens et al., 2008) that may act as potential reservoirs of the virus. Like the other members in the genus *Polerovirus*, TuYV has a genome of a linear, single-stranded, monopartite RNA molecule, encapsulated in an icosahedral shell (LaTourrette et al., 2021).

Because aphids are extremely mobile, reproduce quickly, feed on a broad range of plants, and can travel great distances, they are able to transmit novel virus genotypes and species between regions and countries (Jayasingha et al., 2022). It would be interesting to analyse poleroviruses in Swedish sugar beet samples taken continuously from fields as they would offer important, up-to-date information on the evolution and spread of these viruses. With this knowledge, farmers can minimize crop losses by putting into practice prompt and efficient disease management techniques. Furthermore, developing resistant cultivars and enhancing integrated pest management (IPM) techniques, both of which are critical to sustainable agriculture, require an understanding of how these viruses interact with sugar beet in their natural environments. Still, there is a need for resistant sugar beet cultivars and extended knowledge of virus distribution and diversity would be important in the process of developing resistant cultivars, and disease management with IPM techniques.

1.3. Polerovirus-associated RNAs

Polerovirus-associated RNAs are sub-viral agents that can multiply independently of a helper virus (Schönegger et al., 2022), but they require a helper virus for movement, encapsidation, and vector transmission (Filardo et al., 2021). In general, polerovirus-associated RNA molecules are single-stranded RNA molecules around 2.8 – 3 kb in size with two primary ORFs (Gaafar and Ziebell, 2019). According to recent finding, these RNAs are categorized into the

group tombusvirus-like associated RNAs (tlaRNAs) because of their evolutionary similarities with viruses belonging to the family *Tombusviridae* (Campbell et al., 2020). Their involvement in pathogenicity has not received much attention up to this point. Some polerovirus-associated RNA molecules have been discovered in recent investigations. These include the tobacco vein destroying virus-associated RNA (Tan et al., 2021), pepper vein yellows virus-associated RNA (Schravesande et al., 2021), and carrot red leaf virus-associated RNA (Adams et al., 2014). A study on the carrot virome in Spain and France was able to identify four tlaRNAs and two of those were novel species (Schönegger et al., 2022). Recently, two variants of TuYV-associated RNA were identified in Swedish oilseed rape infected with TuYV: TuYVaRNA and TuYVaRNA2 (Puthanveed et al., 2023). It is not known how common it is with the coinfection of poleroviruses and polerovirus-associated RNA in Swedish crops.

This thesis aimed to identify the variety of aphids-transmitted viruses that infect sugar beet in the southern part of Sweden using different molecular diagnostic techniques. In the previous survey carried out in 2019 (Puthanveed et al., 2023), BMYV, BChV and TuYV were identified in mixed infection. Is that common in general or something specific to 2019? Which viruses are causing VY in Sweden? With this study, it would be possible to obtain a more complete picture.

2 The aim of this study

The first aim of this study was to identify the variety of viruses transmitted by aphids that infect sugar beet in the southern part of Sweden.

The second aim of this study was to test if TuYVaRNA infects sugar beet either together with TuYV or with other poleroviruses.

3 Material and methods

3.1. Plant material

Sugar beet leaves displaying symptoms of virus yellows (Figure 2), like yellowing, had been collected in 2023 by the Swedish Board of Agriculture from seven fields in the county of Skåne for analysis. The samples had been tested with double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for infection by BYV, BtMV and poleroviruses, and samples from four locations (Vadensjö, Lagestorp, Hilleshög and Maglarp) were positive in the test for poleroviruses. The samples from Tågarp tested negative for polerovirus infection in DAS-ELISA, but the absorbance values were just below the threshold for being positive. All samples were negative for infection by BYV or BtMV.

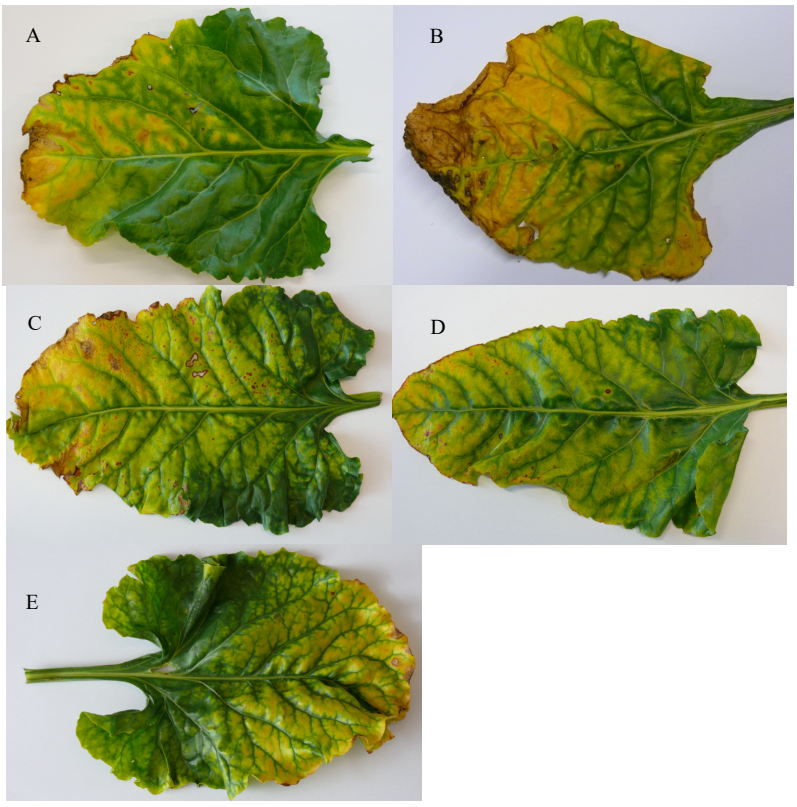

Figure 2: *Symptomatic sugar beet leaves collected from different locations in the county of Skåne and tested by DAS-ELISA for polerovirus infection.* ***A)*** *Vadenjö,* ***B)*** *Lagestorp,* ***C)*** *Hilleshög* ***D)*** *Maglarp,* ***E)*** *Tågarp. The leaves from Tågarp were negative in the DAS-ELISA test, while leaves from the other four locations were positive.*

From each field, 20 random samples had been collected and labelled from 1 to 20 (Table 1). Each sample was labelled during the analysis to correspond with the initial letter of the field name and the sample number (Table 1).

Table 1: Field samples of sugar beet leaves, selected for molecular analyses from Skåne county, Sweden

Plant sample	Location
V3, V4	Vadensjö
L2, L3	Lagestorp
H5, H6, H11, H12	Hilleshög
M1, M2	Maglarp
T3, T4	Tågarp

3.2. RNA extraction

Total RNA was extracted from collected sugar beet leaves (0.1 g per sample) using Sigma Spectrum Plant Total RNA Kit (Sigma-Aldrich), according to the manufacturer's protocol. Plant leaves were ground in liquid nitrogen using an autoclaved mortar and pestle. The finely ground leaf powder was transferred into separate 1.5 ml tubes. In the RNA extraction protocol, after step 3, step 4a was followed to bind RNA into the column. Total RNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

3.3. Gel electrophoresis

RNA integrity was verified by running a gel just after extracting RNA with 2 µL of RNA extract per sample. For gel electrophoresis, 1% agarose gel with 1 x Tris-acetate-EDTA buffer (TAE) was used. To stain nucleic acids, 2 µL of Midori Green (Nippon Genetics) was added to each 50 ml gel. The loading dye applied was TriTrack DNA Loading Dye (6x) from Thermo Fisher Scientific. Thermo Fisher Scientific's Gene Ruler 1 kb DNA Ladder was utilized for

size estimates. The ladder was mixed with loading dye and water according to the manufacturer's protocol.

3.4. cDNA synthesis

Extracted RNA (1 µg) was reverse-transcribed into complementary DNA (cDNA) using RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific) according to the manufacturer's protocol with random hexamer primers.

3.5. PCR

For PCR amplification of the CP gene of poleroviruses, Luteo F and Luteo R (Abraham et al. 2006) were utilized as forward and reverse primers. The expected amplicon size when using these primers is 635 bp. The PCR was tried with both DreamTaq and Phusion polymerase.

Each PCR test was run in a total volume of 20 µL containing DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) or Phusion High-fidelity PCR master mix with HF buffer (Thermo Fisher Scientific), 0.25 µM each of Luteo F and Luteo R primers, 1 µL of cDNA and nuclease-free water. For both Dream Taq and Phusion polymerase the PCR program was 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 20 s and finally 72 °C for 5 min before cooling down. In the negative control, templates were replaced with water.

The amplified products were analyzed on an agarose gel to ensure that the amplicons had the expected size for the CP gene (see section 3.3). For every sample, 5 µL of the PCR product was utilized for verification and the rest was stored at -20 °C.

3.6. Purification of PCR products

RT-PCR products were purified using a GeneJET purification kit (Thermo Fisher Scientific) following the manufacturer's protocol without step 2. Purified products were kept at -20 °C in the freezer. After purification, 5 µL of purified RT-PCR product was used to run a gel to verify their purity and estimate concentration.

3.7. Ligation of purified RT-PCR products with cloning vector

The ligation was performed using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Sticky-end cloning was used for RT-PCR products generated by DreamTaq and blunt-end cloning for products generated by the Phusion polymerase enzyme, respectively, according to the manufacturer's instructions and using a molar ratio of 1:3 for vector and insert. For purified RT-PCR products with a high concentration, 4 µL was used for ligation while 7 µL was used for purified RT-PCR products with low concentration.

3.8. Transformation

For the transformation, Subcloning Efficiency™ DH5α Competent Cells (Invitrogen) of *Escherichia coli* were used according to the manufacturer's protocol. From each ligation, 5 µL was used for the transformation.

From the solution with transformed cells, 100 µL was first spread on an LB agar plate supplemented with 50 µg/ml ampicillin. The remaining solution of transformed cells was centrifuged for three minutes at 225 rpm, and the supernatant was decanted. The bacterial cells were resuspended and spread on a separate LB-agar plate supplemented with 50 µg/ml ampicillin. The plates were incubated overnight at 37 °C. As a control, cells transformed with pUC19 DNA (0.25 ng) were used. The following day, the colonies were counted, and the transformation efficiency was calculated using the control plate.

3.9. Cultivation of transformed cells

Single colonies were selected from the plates with transformed cells and transferred to tubes containing 3 ml of liquid LB medium with 50 µg/ml ampicillin. The selected colonies were also streaked on LB plates containing 50 µg/ml ampicillin. Both liquid cultures and plates were incubated overnight at 37 °C with shaking at 200 rpm for liquid cultures.

3.10. Plasmid DNA purification

From overnight cultures, 1.5 ml of bacterial solution was transferred to a 1.5 mL Eppendorf tube, which was centrifuged at 8000 rpm for 3 min. The supernatant was carefully removed and the rest of the overnight culture (1.5 mL) was added into the same tube and the

centrifugation was repeated. The pellet was subjected to plasmid DNA purification using Gene JET Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Step 7 of the purification process was not included, and there was no second elution. A NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) was used to quantify the yield of plasmid DNA.

3.11. Restriction digest of purified plasmids using *Bgl*II

The restriction enzyme FastDigest *Bgl*II (Thermo Fisher Scientific) was used to digest the plasmids to confirm that the clones had inserts of the expected size. Each reaction contained 1.5 µL of *Bgl*II restriction enzyme, 1 µL of Fast digestion buffer, 2.5 µL of water, and 10 µL of plasmid extract (mentioned in section 3.10.), and the restriction digests were analyzed by gel electrophoresis (see section 3.3) to verify the presence of an insert of the expected size.

3.12. Sequencing

For each isolate, three clones with the expected insert size were Sanger sequenced at Macrogen Europe (Table 2) using pJET1.2 forward and reverse sequencing primers.

Table 2: Clones sent for Sanger sequencing

Sample name	Clone
M1/ Maglarp	a,c,d
H6/ Hilleshög	a,c,d
T3/ Tågarp	a,c,d
L2/ Lagestorp	b,c,d
V3/ Vadensjö	a,c,d

3.13. Sequence analyses

The primer sequences were eliminated before additional analyses. To find the highest nucleotide identities to accessions in GenBank, the Basic Local Alignment Search Tool

(BLAST) was utilized (BLASTn; https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi). The respective sequences were translated and submitted to BLASTp to identify the encoded protein. The Sequence Demarcation Tool (SDT) was used to calculate the pairwise nucleotide sequence identities, which were visualized as a matrix. For the phylogenetic analysis, polerovirus isolates identified in this study, previously sequenced Swedish isolates (Puthanveed et al., 2023) and reference isolates from GenBank were selected and all the sequences were aligned with the Clustal W algorithm in MEGA version X (Kumar et al., 2018). The maximum-likelihood method and the Tamura-Nei model in MEGA version X (Kumar et al., 2018) were used to construct an unrooted phylogenetic tree with 1000 bootstrap replications for the validation of phylogenetic tree branches.

3.14. Primer design for TuYVaRNA

General primers for PCR amplification of TuYV-associated RNA (TuYVaRNA) were designed using BLAST (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi). Sequences of four selected accessions were employed in the alignment: TuYVaRNA and TuYVaRNA2 from Swedish oilseed rape (OP719312, OP719313), Australian TuYVaRNA in rapeseed (MT642443.1), and German TuYVaRNA in pea (MN497834.1). By considering the melting temperature (Tm), GC content, amplicon size, and targeted genome region, three primer pairs (pp1, pp2 and pp3) were selected (Supplementary Table 1). For the three primer pairs, the Tm was 59-60 °C and they had a GC content of 50-55% (pp1 and pp2) and 60-63 % (pp3). The three selected primer pairs amplify overlapping regions within the genome of TuYVaRNA and TuYVaRNA2 (Figure 3) yielding products of 559 bp (pp1), 611 bp (pp2) and 772 bp (pp3).

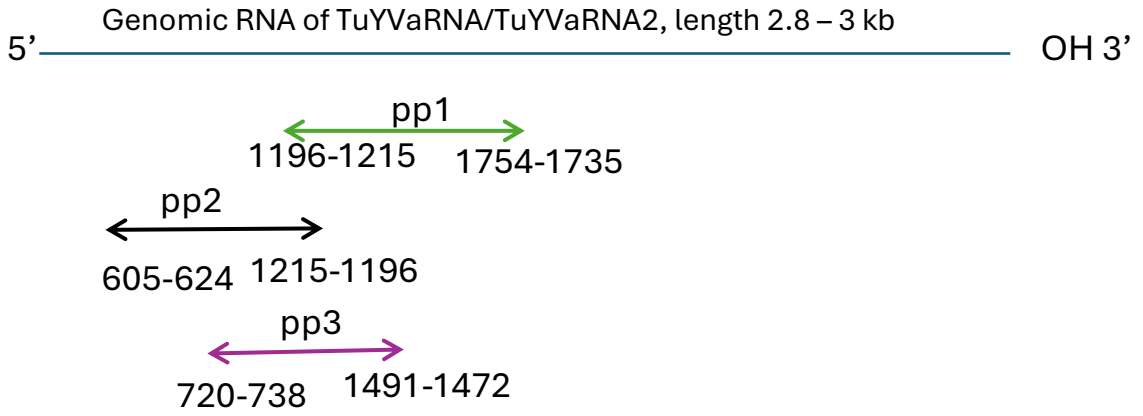

Figure 3: Genomic map for RNA of TuYVaRNA/ TuYVaRNA2. The nucleotide positions of the designed primers and the amplified regions are shown: pp1- primer pair one; pp2- primer pair 2; pp3- primer pair 3.

3.15. RT-PCR for TuYVaRNA

RT-PCR for amplification of TuYVaRNA genomic regions using primer pairs pp1, pp2, and pp3 under section 3.14 was carried out using Phusion polymerase as described in section 3.5 with cDNA of five sugar beet samples (L2, V3, H6, M1 and T3). In addition, all the PCR tests with TuYVaRNA primers were repeated at a lower annealing temperature (50 °C).

4 Results

Sugar beet leaves of plants with suspected VY had previously been tested for virus infection by DAS-ELISA and in this thesis, samples positive for polerovirus, as well as one sample from Tågarp with DAS-ELISA reading close to being positive, were tested further by RT-PCR.

4.1. RNA extractions from leaf samples

Table 3: RNA concentration and A260/280, A260/230 ratio measured after RNA extraction

Location	Sample name	RNA conc. (ng/µl)	A260/280	A260/230
Vadensjö	V3	285.9	2.12	2.42
	V4	270.2	2.10	2.41
Lagestorp	L2	943.3	2.11	2.44
	L3	282.6	2.11	2.29
Hilleshög	H5	341.1	2.10	2.42
	H6	153.8	2.13	2.10
	H11	592.2	2.13	2.43
	H12	597.3	2.13	2.40
Maglarp	M1	309.7	2.11	2.46
	M2	622.6	2.13	2.47
Tågarp	T3	605.7	2.10	2.35
	T4	322.6	2.09	2.29

RNA was extracted from symptomatic leaves of sugar beet from five locations in the county of Skåne with two or four leaf samples per location (Table 3). For all samples, the extracted RNA had a relatively high concentration above 150 ng/µl (Table 3). The extracted RNA had a high absorbance ratio above 2 for both A260/280 and A260/230 (Table 3) and these values were above the normal level, which could be due to impurities.

To check the integrity, the extracted RNA was analyzed by agarose gel electrophoresis (Figure 4). The extracted RNA did not show two separate bands for the respective two subunits present in ribosomal RNA (rRNA), and a smear of low molecular size was visible in the gel indicating partial degradation of RNA (Figure 4).

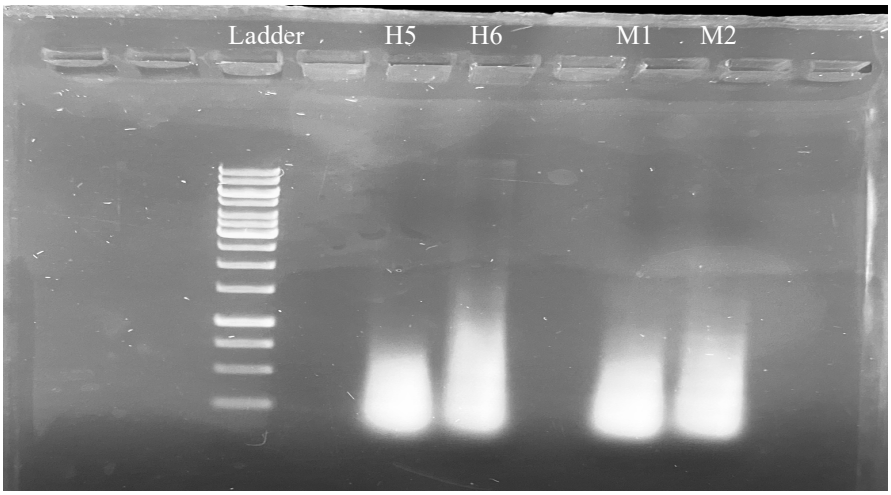

Figure 4: Gel electrophoresis after RNA extraction from sugar beet leaves from different locations: Hilleshög (H5, H6) and Maglarp (M1, M2). Ladder: gene ruler 1kb DNA ladder.

4.3. RT-PCR for detection of poleroviruses

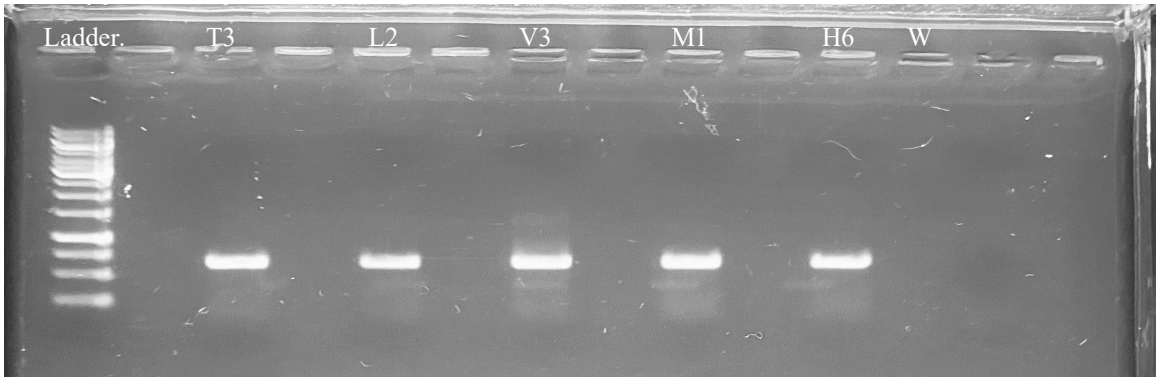

Figure 5: Gel electrophoresis after RT-PCR for detection of poleroviruses with Luteo F and Luteo R primers and Phusion master mix. Ladder: gene ruler 1kb DNA ladder; samples: T3, L2, V3, M1 and H6, water (W) was used as a negative control. The expected band size was 635 bp.

To amplify the gene encoding the CP for poleroviruses, extracted RNA of the samples was reverse-transcribed into cDNA, and the cDNA was subjected to RT-PCR using the primer pair Luteo F and Luteo R together with Dream Taq green PCR master mix. All the 12 tested samples gave amplicons of the expected size (635 bp), except sample H5 (Supplementary Figure 1). The presence of expected-size amplicons confirmed infection with polerovirus. No PCR bands were present in the negative control (Supplementary Figure 1). Because of the absence of the expected size band in sample H5, it was not used for further analysis. The same results in RT-PCR were obtained for these samples when using the Phusion master mix with

Luteo F/Luteo R (Figure 5). The two samples from location Tågarp (T3 and T4) turned out to be positive with RT-PCR even if previously testing negative in DAS-ELISA.

4.4. Cloning

4.4.1. Purification of RT-PCR products

RT-PCR products generated both by Phusion and Dream Taq with the expected band size for 11 positive samples were separately purified and to confirm the successful purification, a gel was run. A single clear band of the expected size was observed with different levels of brightness (Figure 6, Supplementary Figure 2).

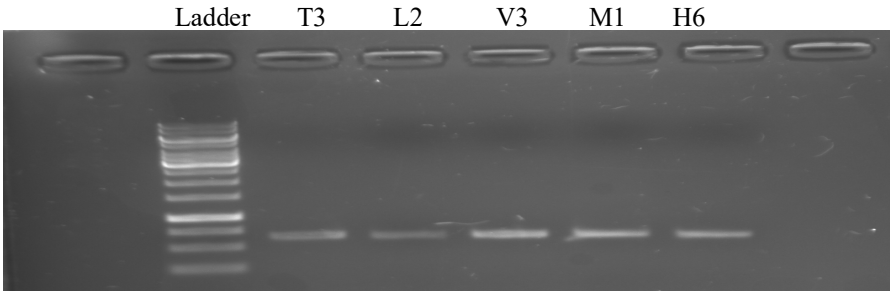

Figure 6: Gel electrophorosis after purification of PCR products generated with Phusion. Ladder: gene ruler 1kb DNA ladder; samples: T3, L2, V3, M1 and H6.

4.4.2 Ligation of RT-PCR products and transformation

Cloning was first attempted with purified RT-PCR products amplified using Dream Taq polymerase and pJET 1.2 blunt end vector following the sticky end protocol. To calculate transformation efficiency, transformation was also done using pUC19 plasmid, where a value above 10^6 transformants/µg DNA indicates a successful transformation. However, the colony count obtained for ligated Dream Taq polymerase products after transformation (Supplementary Table 2) was very low, though the transformation efficiency was high (5.76 x 10^6 transformants/µg DNA). The transformation was repeated twice and the same low number of clones were obtained. Therefore, the cloning attempts were repeated with PCR products generated with Phusion.

Using the pJET 1.2 blunt end vector, the blunt end protocol with Phusion PCR product was followed for the ligation. The ligation products were transformed into competent cells of *E. coli* DH5α.

Table 4: Number of bacterial colonies on LB plate after transformation

Sample	100 µl culture	Remaining solution
T3	12	135
L2	-	9
V3	1	21
M1	13	48
H6	37	115
pUC19	144	overgrowth

The obtained transformation efficiency was 11.36 x 10^6 transformants/µg DNA for this transformation and it was a positive result for this type of competent cells. This time, a sufficient number of colonies were produced for all samples (Table 4). Four colonies were picked for each sample and used to start overnight cultures for plasmid purification.

4.5. Restriction enzyme digest analysis of plasmid DNA

Plasmid DNA was isolated from overnight cultures of selected colonies from the transformation. By using the restriction enzyme *Bgl*II to digest the purified plasmids, the size of the insert was determined. DNA fragments with the expected size of 635 bp from RT-PCR with universal primers that target the CP gene of poleroviruses were successfully cloned into a plasmid for all five samples (Figure 7).

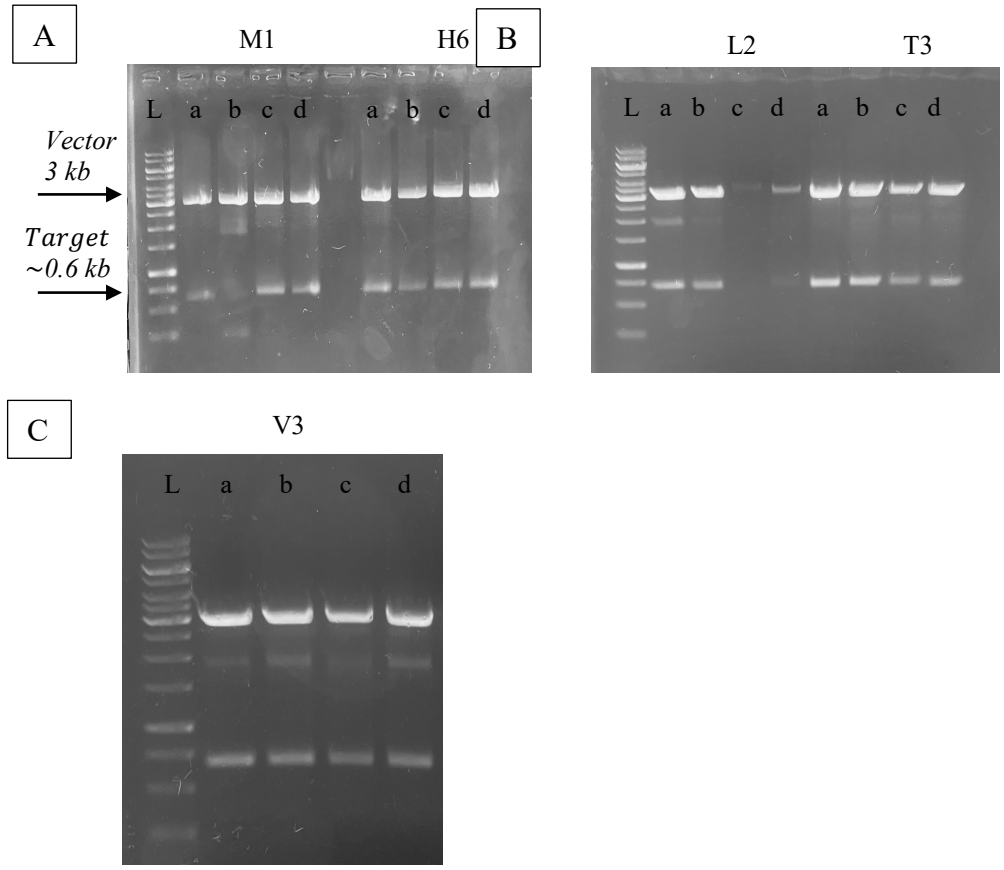

Figure 7: Gel electrophoresis of plasmid DNA digest with BglII. Ladder/L: gene ruler 1kb DNA ladder. ***A)*** *clones M1a-M1d and H6a-H6d,* ***B)*** *clones L2a-L2d and T3a-T3d,* ***C)*** *clones V3a-V3d.*

All four clones for samples H6, V3, and T3 contained inserts with the expected size (Figure 7). Clone M1b did not contain an insert of the expected size while the other three M1 clones did (Figure 7A). The four clones for L2 had inserts of the expected size, but two of them had low concentrations of plasmid DNA (Figure 7B).

4.7. Sequence comparison

For each sample, three clones were selected for sequencing and the sequence for all clones resulted in chromatograms with good signal and clear peaks. The primers Luteo F and Luteo R were identified in the clone sequences, except for clone H6A, which lacked primer Luteo F. The length of the PCR product was 635 bp and for the analyses, the primer sequences at the ends of the PCR product were removed and the sequence for subsequent analysis was 581 bp. The pairwise sequence comparisons showed that the three clones of isolate M1 were identical and that this was also found for the clones of H6, V3 and L2. The insert sequence of clone H6a

was 555 bp which was slightly shorter than H6c and H6d. Clones of T3 were not identical in sequence, but they had an identity of 99% (Supplementary Table 3).

In BLASTn search of GenBank, the three clones for each sample showed the highest nucleotide sequence identity with the CP gene of BMYV or BChV isolates (Table 5). Clones of T3 shared the highest nucleotide sequence identity at 97% with a Polish BMYV isolate (Accession number EU148508) and the clones of H6 showed the highest sequence identity at 99% with a Swedish BMYV isolate (OP719306) (Table 5). All the sequenced clones of L2, V3 and M1 showed the highest sequence identity to a French BChV isolate (EU022510) (Table 5). A conclusion that can be made according to the result of GenBank nucleotide search is that the BMYV isolate H6 in this study had the highest nucleotide identity with an earlier identified Swedish BMYV isolate, while the other isolates had the highest nucleotide identity to virus isolates from other countries (Table 5).

According to the BLASTp search with translated sequences of the protein database (Supplementary Table 4), the cloned sequences coded for the CP and the movement protein of the respective viruses identified in the BLASTn search (Table 5).

Table 5: Result of BLASTn search of GenBank with sequences of cloned PCR products

Clone	Description of accession with highest sequence identity	Accession number	Identity (%)
T3a	Beet mild yellowing virus isolate 19K CP-RT fusion protein and coat protein genes, complete cds	EU148508	97.07
T3c	Beet mild yellowing virus isolate 19K CP-RT fusion protein and coat protein genes, complete cds	EU148508	97.24
T3d	Beet mild yellowing virus isolate 19K CP-RT fusion protein and coat protein genes, complete cds	EU148508	97.24
L2b	Beet chlorosis virus isolate M26 CP protein and RT protein genes	EU022510	99.83
L2c	Beet chlorosis virus isolate M26 CP protein and RT protein genes	EU022510	99.83
L2d	Beet chlorosis virus isolate M26 CP protein and RT protein genes	EU022510	99.83
V3a	Beet chlorosis virus isolate M26 CP protein and RT protein genes, complete cds	EU022510	99.66
V3c	Beet chlorosis virus isolate M26 CP protein and RT protein genes, complete cds	EU022510	99.66
V3d	Beet chlorosis virus isolate M26 CP protein and RT protein genes, complete cds	EU022510	99.66
M1a	Beet chlorosis virus isolate M26 CP protein and RT protein gene	EU022510	99.83
M1c	Beet chlorosis virus isolate M26 CP protein and RT protein genes	EU022510	99.83
M1d	Beet chlorosis virus isolate M26 CP protein and RT protein genes	EU022510	99.83
H6a	Beet mild yellowing virus isolate Alnarp_Lu22b coat protein gene	OP719306	99.82
H6c	Beet mild yellowing virus isolate Alnarp_Lu22b coat protein gene	OP719306	99.83
H6d	Beet mild yellowing virus isolate Alnarp_Lu22b coat protein gene	OP719306	99.83

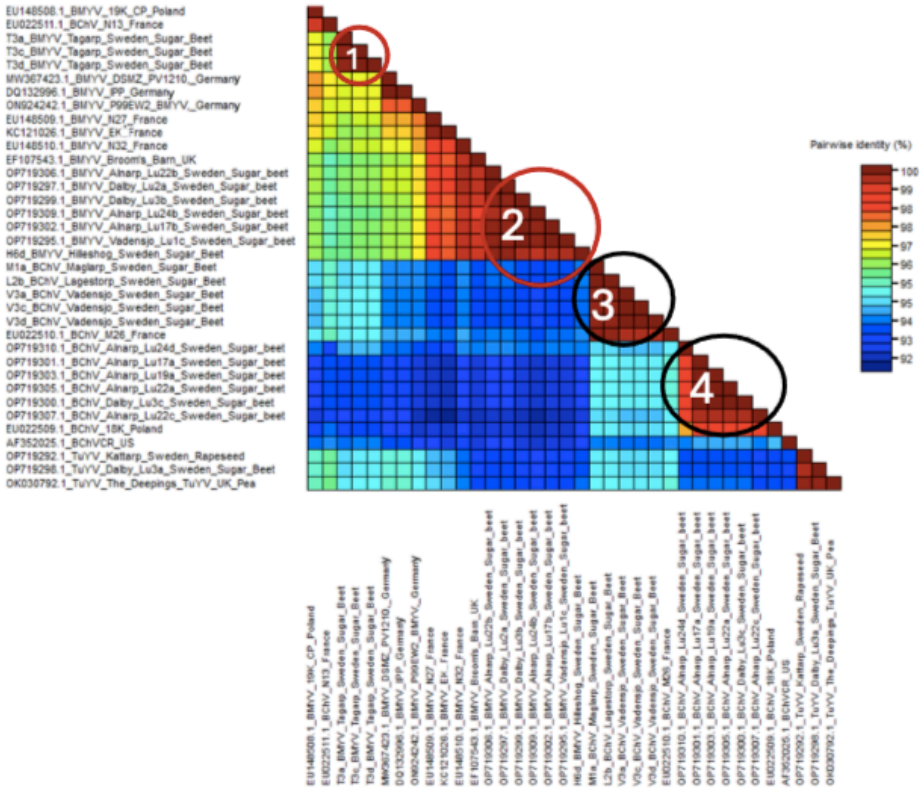

Figure 8: Pairwise identity matrix of polerovirus coat protein gene sequences of Swedish isolates from sugar beet and reference isolates selected from GenBank. Clones are named as clone name, virus name, location, and host: the references are named as accession number, virus name, location, and host (when not sugar beet). Red circle 1: BMYV isolate identified in the current study; Red circle 2: BMYV isolates identified earlier in Sweden with BMYV H6d identified in this study; Black circle 3: BChV isolates identified in the current study; Black circle 4: BChV isolates identified earlier in Sweden.

Pairwise identities between the identified and selected sequences from GenBank were calculated using SDT, and the results were shown as matrices (Figure 8). Reference isolates from GenBank were selected due to their distinct geographic origin and varying degrees of relatedness to the previously sequenced isolates of BMYV and BChV from Sweden (Puthanveed et al., 2023). For the analysis, representative clones were used for the isolates of Hilleshög, Maglarp and Lagentorp because the clones were identical in sequence.

Two isolates were identified as BMYV in the current study (Hilleshög 6 and Tågarp 3) and one of the sequenced clones for the isolate of Hilleshög is BMYV H6, which shared the highest identity at 99.7 - 99.8% with previously sequenced Swedish isolates of BMYV (accession number OP719309, OP719299, OP719302, OP719295, OP719306, OP719297) (Figure 8). For the other isolate of BMYV identified in the current study (Tågarp 3), the T3 clones had the

highest identity with GenBank accession EU148508.1 BMYV Poland (96.9 – 97.1%) while the second highest identity was with accession EU022511.1 BChV France (96.4%).

All of the BChV clone sequences of the current study (isolates M1, L2 and V3) shared the highest sequence identity with each other and lower sequence identity with previously sequenced Swedish isolates of BChV (OP719310, OP719301, OP719307, OP719303, OP719305, OP719300). BChV clones sequenced during this study had the highest identity with the GenBank accession EU22510.1 BChV-M26 France at 99.7 - 99.8% (Figure 7).

4.8. Phylogenetic analysis

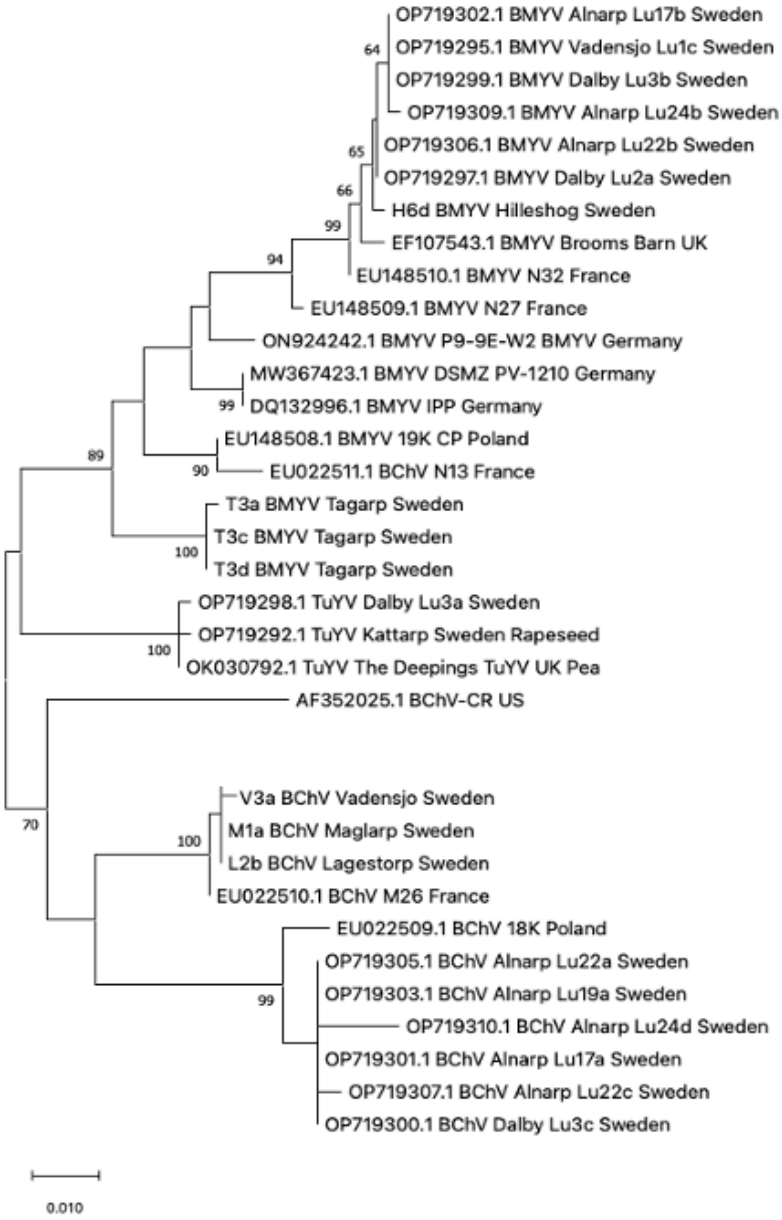

Figure 9: Maximum likelihood tree of coat protein gene sequences from Swedish isolates of BMYV and BChV. Reference isolates of BMYV, BChV and TuYV were included in the analysis. Reference isolates are named by the GenBank accession number, virus, geographic origin, and host (when not sugar beet). The same way of naming was followed for the isolates sequenced in this study without accession numbers. The branch values represent the bootstrap values (1000 iterations) exceeding 60%. The scale shows nucleotide substitutions per site.

To illustrate the relationship between the Swedish isolates and selected isolates from GenBank, a phylogenetic tree was constructed using the maximum likelihood method and the Tamura-Nei model (Kumar et al., 2018). Clones of each of the isolates M1, H6, V3 and L2 were

identical in sequence (Supplementary Table 3), and only one clone from each isolate was used for the phylogenetic tree construction.

The sequenced clones grouped with either BMYV or BChV (Figure 9). In group BMYV, clones of isolate T3 formed a well-supported group (bootstrap value 100), and they were separated from the earlier sequenced Swedish isolates of BMYV. The sequence of isolate H6-BMYV was closely related to those of the reference Swedish isolates of BMYV as well as the isolates BMYV Brooms Barn UK (EF107543) and BMYV N32 France (EU148510). All these isolates formed a well-supported clade with a bootstrap value of 99.

Within the group BChV, the sequenced isolates from this study were separated from the previously sequenced Swedish BChV isolates and they had a close relationship with the M26 isolate from France (EU022510) (bootstrap value 100).

Based on the phylogenetic analysis, the new BChV isolates were different from isolates previously identified from Sweden and this differs from the results for BMYV, where one isolate (H6) was closely related to previously sequenced Swedish BMYV isolates, while isolate T3 was clearly separate (Figure 9).

4.9. RT-PCR test for TuYVaRNA

Primers were designed for detection of TuYVaRNA by RT-PCR. Using four sequences of TuYVaRNA available in GenBank, three primer pairs were designed: pp1, pp2 and pp3. For PCR, cDNA of samples L2, V3, H6, M1 and T3 was tested with two different annealing temperatures at 55 °C and 50 °C. None of the samples tested with RT-PCR was able to give the expected band with the respective primer pair (Supplementary Figure 3) with the annealing temperature of 55 °C.

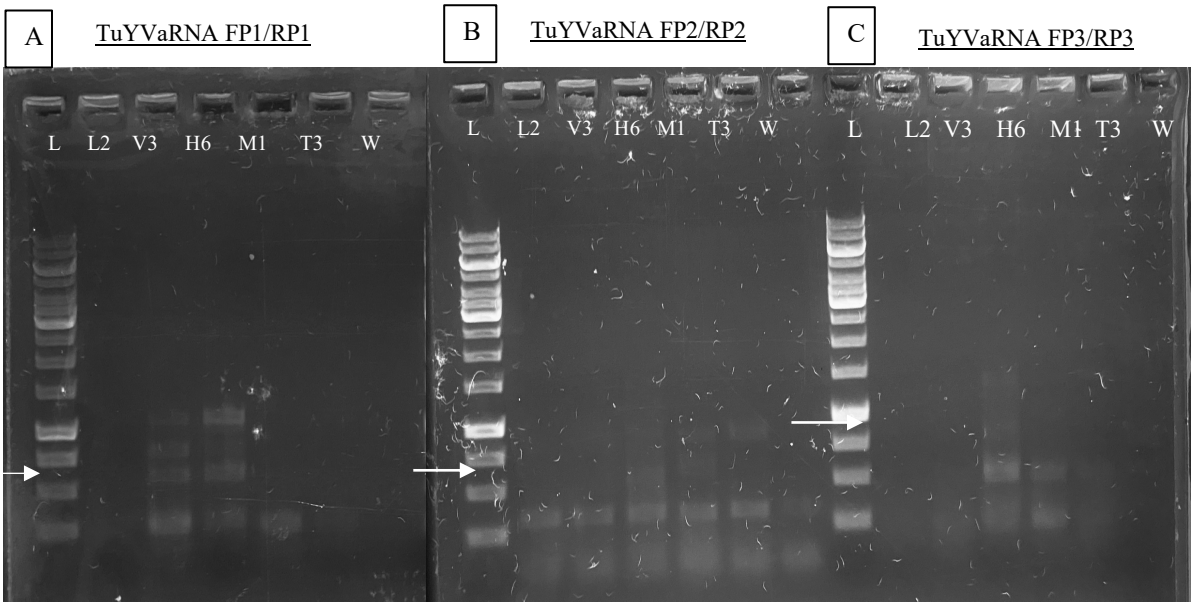

*Figure 10: Gel electrophoresis of testing for the presence of TuYVaRNA in field samples of sugar beet with RT-PCR and three primer pairs using an annealing temperature of 50 °C. Ladder: gene ruler 1kb DNA ladder. **A**) TuYVaRNA primer pair 1 with the expected band size 559 bp, **B**) TuYVaRNA primer pair 2 with the expected band size 611 bp, **C**) TuYVaRNA primer pair 3 with expected band size 772 bp. For RT-PCR, RNA of samples L2, V3, H6, M1 and T3 was used with water (W) as a negative control. Arrows show the expected band size for each primer pair.*

For all three primer pairs, RT-PCR with the annealing temperature of 50 °C resulted in multiple small fragments (Figure 10). The products of expected band size as well as additional smaller products were observed with all three primers and with different tested samples. The brightness of the bands was very low (Figure 10).

5 Discussion

5.1. Two poleroviruses were identified in sugar beet samples

The secret to effective disease control, particularly in the context of sustainable agricultural methods, is accurate diagnosis of the causing agents. Worldwide, the sugar beet crop has suffered severe output loss due to a high disease level of VY (Hossain and Varrelmann, 2021). BMYV, BChV and BWYV are the three common and widespread members of the family *Solemoviridae* that are causal agents of VY in sugar beet (Stevens et al., 2005a). Understanding the diversity of viruses transmitted by aphids that infect sugar beet in the southern region of Sweden was one of the objectives of this study. By using RT-PCR amplification with the universal primer pair Luteo F and Luteo R (Abraham et al., 2006), electrophoresis, and Sanger sequencing, it was possible to identify two poleroviruses present in sugar beet leaves with symptoms of VY collected from the county of Skåne by the Swedish Board of Agriculture.

5.2. A new variant of BMYV and BChV based on the CP gene

All the samples tested seemed to be infected either by BMYV or BChV. The existence of BMYV and BChV independently, but not as a mixed infection, was identified by sequencing of cloned amplification products with three clones per leaf sample. Occurrence of viruses causing VY is naturally fluctuating in Europe, with BMYV being dominant in the northern and western areas and BYV more common in the Mediterranean region (Stevens et al., 2005b). In the current study, BChV was identified in three out of five tested samples (60%) and two out of five samples (40%) were infected by BMYV. A previous field survey in Sweden using symptomatic sugar beet leaf samples demonstrated mixed infection of three poleroviruses, identified as TuYV, BChV and BMYV (Puthanveed et al., 2023). Nevertheless, TuYV was not found in any of the analyzed samples in the present thesis. In the current study, samples from Tågarp that were previously negative in ELISA test for polerovirus were positive for BMYV when using RT-PCR. This means that RT-PCR is more sensitive for virus detection than DAS-ELISA.

Upon examination of the CP gene, the BChV isolates examined in this thesis showed a higher degree of identity with one another than with isolates that had been previously identified

in Sweden. The Hilleshög isolate H6, for which all three clones were identified as BMYV, had the highest sequence identity to previously sequenced Swedish BMYV isolates from sugar beet (Puthanveed et al., 2023). Isolate T3, which also was identified as BMYV, was phylogenetically distinct from the BMYV isolates found in previous studies. Clones of T3 were very similar to each other in sequence and clustered together in the phylogenetic analysis with a 100 bootstrap value. The T3 isolate had the highest sequence identity to BMYV Poland (Kozlowska-Makulska et al., 2007) and BChV N-13 France. BChV N-13 France is a recombinant virus that has more similarities with BMYV based on the CP gene and with BChV based on the P0 gene (Kozlowska-Makulska et al., 2015). Research by Kozlowska-Makulska et al., (2015) further revealed that beet polerovirus populations have recombinants harbouring breakpoints within the CP gene. It will be necessary to examine thoroughly the complete genome sequence of the T3 isolate to get a clear idea about how it differs from other sequenced BMYV isolates, but based on this thesis it is clear that the BMYV isolate found here (Tågarp 3) is a new variant.

The BChV isolates studied in this thesis (V3, M1, L2) were found to be closely related to BChV isolate M26 from sugar beet in France (EUO22510.1) (Kozlowska-Makulska et al., 2007). Though the three BChV isolates identified in this study clustered together in the phylogenetic analysis, they also shared high nucleotide identity with previously identified Swedish sugar beet BChV isolates (Puthanveed et al., 2023). The phylogenetic tree clearly showed that the BChV isolates found in this study were different from isolates found previously in Sweden. This can be a separate introduction to Sweden with a different BChV genotype.

This thesis was conducted to analyse the diversity of poleroviruses and found what might be single infections with novel variants of BMYV and BChV in the tested sugar beet samples. In the previous Swedish survey from 2019 (Puthanveed et al., 2023), mixed infection of TuYV, BMYV and BChV was recognized in sugar beet plants. Mixed infection of poleroviruses can lead to a high risk of emerging new virus variants after recombination (Kozlowska-Makulska et al., 2015). Therefore, mixed infections may be the cause of the newly developing viral variants. At the same time, the survey of 2019 might have missed genotypes that were present in Sweden at a low frequency and they may now have become more common. The climatic change occurring all around the world may lead to changes in aphids' lifestyles and influence them to migrate. Those migrating aphids can also bring different virus variants from one place to another (Krishnareddy, 2013) leading to the emergence of new virus variants. In addition, limited possibilities to manage aphids after the ban on neonicotinoids could also be an indirect reason for new virus variants emerging.

5.3. Search for polerovirus-associated RNA molecules

It has been discovered that associated RNAs are present together with poleroviruses (Gaafar and Ziebell, 2019), but finding polerovirus-associated RNA in the examined sugar beet samples proved to be difficult. For this study, primers were designed based on sequences of TuYVaRNA and TuYVaRNA2 because according to the previous study, Puthanveed et al. (2023) were able to detect TuYVaRNA and TuYVaRNA2 in Swedish oilseed rape and TuYV in Swedish sugar beet plants. Based on these results, this study hypothesized that TuYVaRNA/ TuYVaRNA2 infects sugar beet either together with TuYV or with other poleroviruses. For RT-PCR amplification, sequences of four TuYVaRNA/ TuYVaRNA2 isolates identified from Sweden, Australia, and Germany were used to design new primers (Puthanveed et al., 2023, Filardo et al., 2021). Three primer pairs were designed and tested to increase the possibility of detecting TuYVaRNA. It is possible that associated RNA molecules were not discovered because the primers did not bind to the associated RNA, that associated RNAs were not present in the studied samples, the amount of associated RNA might be under the detection level, the RT-PCR procedure was not sensitive enough to identify associated RNA molecules or the tested sample might be infected with other polerovirus-associated RNA molecules, but not with TuYVaRNA/TuYVaRNA2. If we could test the developed primers using samples that were already infected with TuYVaRNA, primer specificity might be investigated. Due to time constraints, it was difficult to experiment with optimizing the RT-PCR procedure using various annealing temperatures and primer combinations as well. It was possible to obtain amplicons for certain samples at the 50 °C annealing temperature. Numerous bands suggest that additional purification, cloning, and sequencing processes may be needed to see if the band of expected size or other bands are amplification products of TuYVaRNA. The previous identification of associated RNA in oilseed rape was successful with the high throughput sequencing (HTS) technique (Puthanveed et al., 2023). Therefore, given the outstanding specificity of this method, we should be able to identify associated RNA molecules if we were to analyse the samples using HTS.

5.4. General challenges throughout the analysis

The execution and results of the research thesis might have been affected by a set of frequently occurring problems. Some of the main challenges faced throughout the practical part of the thesis are mentioned here. The first and foremost challenge is RNA degradation. In cellular biology, RNA degradation is an essential process that controls gene expression, preserves RNA quality control, and is relevant to every experimental step that is carried out (Grodetskaya et al., 2021). RNA degradation normally occurs before or early during the extraction. The leaves used for RNA extraction had been collected late in the season of 2023 and had then been stored in the freezer. Because of the late collection, the leaves had been aging, and the RNA may have been partially degraded before RNA was extracted. It was important to add the homogenized leaf powder to the buffer solution without thawing it before beginning the RNA extraction procedure. Because of its susceptibility to degradation by RNases after being released from the cell, it was difficult to work with RNA. It would be easy if we could use a stabilizing regent immediately after the extraction of RNA. Even though the gel electrophoresis image did not show two distinct bands for ribosomal RNA, the absorbance ratio values suggested that the RNA might not have been destroyed and there was no problem with the following RT-PCR test. The reverse transcription process can be affected by degraded RNA, leading to fragmented or incomplete cDNA (Opitz et al., 2010). This can lead to partial or inefficient PCR amplification, potentially producing inaccurate results (Tong et al., 1997). The gel tray was thoroughly cleaned before each usage, reducing the risk of RNase contamination during gel electrophoresis. To avoid environmental contamination, sterile benches were used during the PCR sample preparation.

In this study, samples were used from five locations in the region of Skåne, with RT-PCR test of two samples per location and cloning of PCR products from a single sample per location. Consequently, the final result was reached using a small sample size, and the conclusion would be stronger if we could use a larger number of samples in future analyses.

5.5. Future work

The T3 isolate of this study is a new genotype and possibly a recombinant of BMYV and BChV. High-throughput sequencing of the T3 isolate and whole genome sequencing will be

beneficial in revealing the events that led to the T3 isolate's separation from the BMYV isolates that had been previously sequenced in Sweden.

5.6. Conclusion

Every analysed leaf sample seemed to have a single infection by either BChV or BMYV with 40% of the analysed samples being infected with BMYV and 60% with BChV. Based on CP gene sequence, the BChV and BMYV isolates included novel virus genotypes. This could be because of the introduction of new virus genotypes by migrating aphids or by virus recombination. The results show that the diversity of BMYV and BChV in Sweden is higher than previously thought. A lack of effective aphid control measures might provide a lot of opportunity for mixed infections of viruses in plant hosts, which could result in the emergence of new virus genotypes and be a detrimental effect of EU's prohibition of neonicotinoids.

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8 Supplementary materials

8.1. Supplementary tables

Supplementary Table 1: Primers used in this study for amplification of polerovirus genome sequences and TuYVaRNA molecules

Primer	Sequence '5 - 3'	Amplicon Size (bp)
Luteo R	CACGCGTCNACCTATTT NGGRTTNTG	635
Luteo F	GCTCTAGAATTGTTAAT GARTACGGTCG	
TuYVaRNA fwd1	GTTCAAGCGATTTGCAGCCA	559
TuYVaRNA rev1	GACCGGTCTCGTTTGACAGA	
TuYVaRNA fwd2	GAGGTGGTTCGTCGAGGTTA	611
TuYVaRNA rev2	TGGCTGCAAATCGCTTGAAC	
TuYVaRNA fwd3	CCCCGGCACACTAACCCTA	772
TuYVaRNA rev3	CGCCTCTCTTGTGGTACCTG	

Supplementary Table 2: Number of colonies for ligation products of Dream Taq generated amplicons

Sample	100 µl culture	Remaining solution
T3	2	2
L2	1	8
V3	-	1
M1	-	-
H6	-	-
pUC19	144	overgrowth

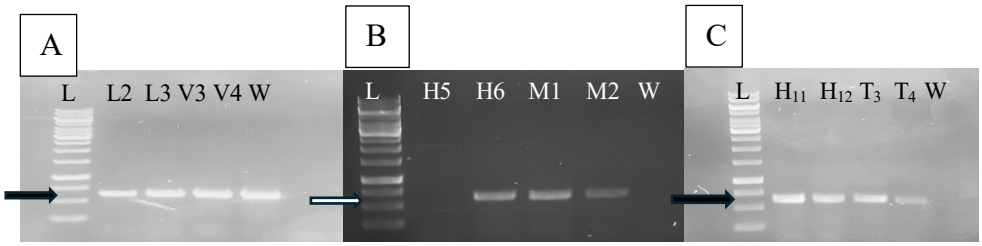
Supplementary Table 3: Comparison of clone sequences for the same sample using BLASTn

Clone name	Sequence identity (%)
M1a-M1c	100
M1a-M1d	100
M1c-M1d	100
H6c-H6d	100
L2b-L2c	100
L2b-L2d	100
L2c-L2d	100
V3a-V3c	100
V3a-V3d	100
V3c-V3d	100
T3a-T3c	99
T3a-T3d	99
T3c-T3d	100

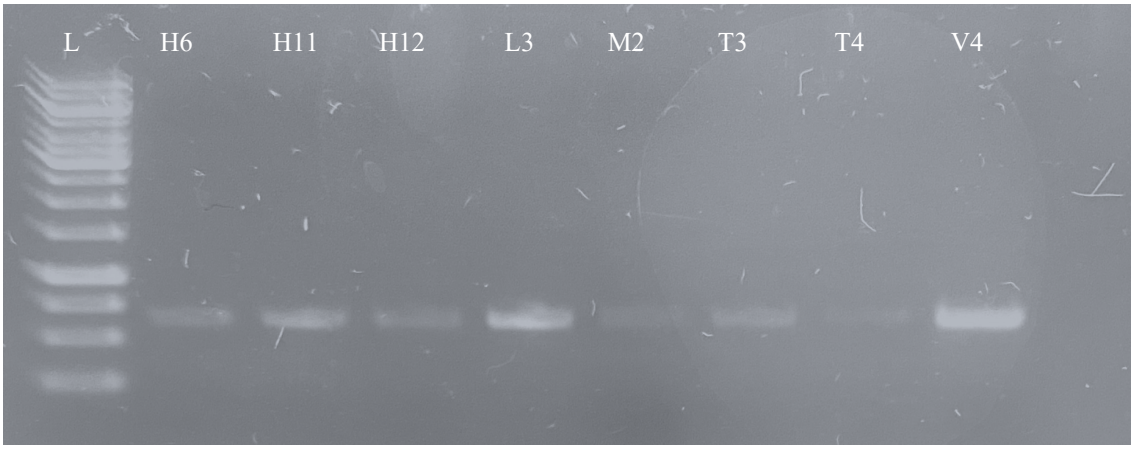
Supplementary Table 4: Results of BLASTp search of protein database with translated sequence of cloned PCR products

Clone name	Description	Sequence ID	Sequence identity (%)
H6a	coat protein, partial (Beet mild yellowing virus)	WDZ04305.1	100.00
H6c	movement protein [Beet mild yellowing virus]	WDZ04306.1	100.00
H6d	movement protein [Beet mild yellowing virus]	WDZ04306.1	100.00
M1a	P4 [Beet chlorosis virus]	WBG54130.1	99.00
M1c	P4 [Beet chlorosis virus]	WBG54130.1	99.00
M1d	P4 [Beet chlorosis virus]	WBG54130.1	99.00
L2b	P4 [Beet chlorosis virus]	WBG54130.1	99.43
L2c	P4 [Beet chlorosis virus]	WBG54130.1	99.43
L2d	P4 [Beet chlorosis virus]	WBG54130.1	99.43
T3a	coat protein, partial [Beet mild yellowing virus]	WDZ04305.1	100.00
T3c	coat protein, partial [Beet mild yellowing virus]	WDZ04305.1	100.00
T3d	coat protein, partial [Beet mild yellowing virus]	WDZ04305.1	100.00
V3a	CP protein [Beet chlorosis virus]	ACA61698.1	100.00
V3c	CP protein [Beet chlorosis virus]	ACA61698.1	100.00
V3d	CP protein [Beet chlorosis virus]	ACA61698.1	100.00

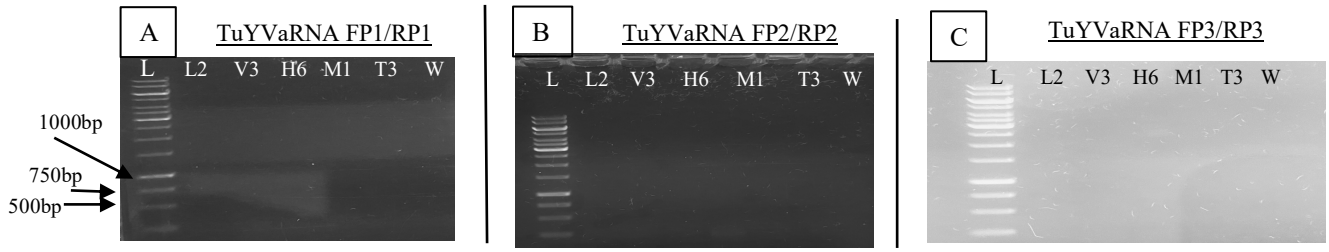
8.2. Supplementary figures



Supplementary Figure 1: Gel electrophoresis after RT-PCR for detection of polerovirus with Luteo F and Luteo R primers and Dream Taq master mix. Ladder/L: gene ruler 1kb DNA ladder. ***A)*** *Samples L2, L3, V3, V4.* ***B)*** *Samples H5, H6, M1 and M2.* ***C)*** *Samples H11, H12, T3, T4. Water (W) was used as negative control. The expected band size was 635 bp which is marked by an arrow.*



Supplementary Figure 2: Gel electrophoresis after purification of PCR products generated with Dream Taq master mix. Ladder: gene ruler 1kb DNA ladder. Samples: H6, H11, H12, L3, M2, T3, T4 and V4.



*Supplementary Figure 3: Gel electrophoresis of testing for the presence of TuYVaRNA in field samples of sugar beet with RT-PCR and three primer pairs using an annealing temperature of 55 °C. Ladder: gene ruler 1kb DNA ladder. **A**) TuYVaRNA primer pair 1 with the expected band size 559 bp, **B**) TuYVaRNA primer pair 2 with the expected band size 611 bp, **C**) TuYVaRNA primer pair 3 with expected band size 772 bp. For RT-PCR, RNA of samples L2, V3, H6, M1 and T3 was used with water (W) as a negative control. Arrows show the size of the bands in the DNA ladder.*

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