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Louise Lidemalm

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Louise Lidemalm

Supervisor: Anders Kvarnheden, Swedish University of Agricultural Sciences, Department of Plant Biology

Examiner: Joel Sohlberg, Swedish University of Agricultural Sciences, Department of Plant Biology

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Sammanfattning

Stritar kan vara bärare av och sprida dvärgvirus vilka sedan många år orsakat skördeförluster inte bara i vetefält och i Sverige utan även hos korn och havre i andra delar av världen. I denna rapport studeras den veteinfekterande typen också kallad vetedvärgvirus som orsakar vetedvärgsjuka. Vetedvärgviruset sprids till nya plantor med vektorn randig dvärgstrit (*Psammotettix alienus*) och kan då orsaka vetedvärgsjuka, symptom av vetedvärgsjuka kan endast ses om infektionen sker i ett relativt tidigt tillväxtstadie hos vete. Symptomen hos infekterat vete är dvärgväxt, gula områden/streck på bladen och minskade huvuden hos axen. Att kunna kontrollera spridningen av vetedvärgvirus är av stort intresse då ungefär 20 % av kalorierna människor konsumerar kommer från vete, då främst brödvetet *Triticum aestivum*. Vete är även en viktig källa till proteiner, vitaminer och mineraler. I denna studie har virus från stritar insamlade i Skåne under 2010 studerats för att se om deras arvsmassa skiljer sig från tidigare analyserade sekvenser. Detta är av intresse eftersom det inte dokumenterats några problem med vetedvärgsjuka i vetefält i södra Sverige på nästan 100 år. Virus-DNAt extraherades från stritarna analyserades med hjälp av molekylärbiologiska metoder såsom PCR, rolling circle amplifiering och klyvning med restriktionsenzym, samt sekvensbestämning varefter sekvenserna jämfördes med sekvenser hämtade ur databasen GenBank. Från de genomförda analyserna kunde det konstateras att vetedvärgviruset var närvarande i stritarna. Från de sekvenser som erhållits kunde det konstateras att stritarna innehöll vetedvärgvirus av stammen WDV-E. Sekvenserna avviker endast marginellt från tidigare isolerade WDV-E virus från Sverige och Europa och det är därför inte troligt att det är en ny genotyp av vetedvärgvirus som hittats i Skåne.

Abstract

Wheat dwarf disease caused by *Wheat dwarf virus* (WDV) can cause a lot of damage in wheat fields by reduction of yield. WDV belongs to the genus *Mastrevirus* in the family of *Geminiviridae* and it is transmitted by the leafhopper *Psammotettix alienus*. Viruses in the genus *Mastrevirus* have a genome of single-stranded (ss) circular DNA ranging in size from 2.6 kb – 2.8 kb. Mastreviruses have monopartite genomes with three genes: a movement protein (MP) gene, coat protein (CP) gene, and *Rep*. Replication-associated protein (Rep) and replication-associated protein A (RepA) are both translated from *Rep*, but from two different mRNAs. For Rep, an intron has been spliced out, while for RepA the intron has been kept. The aim of this study was to see if the WDV isolates from samples collected in Skåne, Sweden during 2010 was a new genotype, by analysing the DNA sequences. The samples from Skåne were of interest because for many years there have been no records of wheat dwarf disease in Skåne. The hypothesis was that there was no new genotype of WDV and thus, that no recombination have occurred. To test the hypothesis DNA was extracted from leafhoppers collected at different locations in Skåne and analysed with PCR, rolling circle amplification and digestion with restriction enzymes. To be able to use the total DNA for analysis a new method for extracting the DNA from leafhoppers was tested, which was verified to be successful. Alignments and a phylogenetic analysis were performed in MEGA 5.1 of the sequences obtained from the analysed samples, sequences from GenBank, and the master thesis by Yazdkhasti (2012). From the sequence analysis it could be confirmed that WDV from the wheat strain WDV-E was present in the samples with a high nucleotide identity so the conclusion was drawn that the isolates are close relatives to isolates from Sweden, China, and the rest of Europe. From the sequence and phylogenetic analyses it could be seen that it was likely that there had been no major recombination in the genome and that it was no new genotype of WDV.

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1. Introduction

1.1 Background

Wheat dwarf virus (WDV) is found in the family *Geminiviridae* in the genus *Mastrevirus* (Ramsell et al., 2008). The family *Geminiviridae* is divided into four genera, based on the viral genome organisation, host range and the vector (Boulton, 2002). The four genera are *Begomovirus*, *Curtovirus*, *Mastrevirus*, and *Topocuvirus.* The morphology of the geminivirus virions is typically twinned or so called geminate. The genome of geminiviruses consists of single-stranded (ss) circular DNA with a genome size ranging from 2.5 kb – 3.0 kb, except for begomoviruses that can have a genome with two DNA molecules, with a total size of 5.5 kb (Brown et al., 2012).

Mastreviruses have monopartite genomes, which means that the genome is found on only one molecule of nucleic acid (Gelderblom, 1996) with a size ranging from 2.6 kb to 2.8 kb. In order to replicate, a double-stranded (ds) DNA version of the genome is formed. It is used as a template for transcription that will give a complementary- (C) and a virion (V) sense transcript. From the transcripts four conserved proteins are translated. Coat protein (CP) and movement protein (MP) are produced from the V-sense transcript, while replication-associated protein (Rep), and replication-associated protein A (RepA) are produced from the C-sense transcript. Rep and RepA are required early in the infection cycle since they are associated with the viral replication (Boulton, 2002). The two proteins Rep and RepA originate from one and the same gene *Rep*, which can be translated into the two proteins through differential splicing (Ramsell et al., 2008). The mRNA translates into Rep after an intron has been spliced out, whereas RepA is translated from an unspliced mRNA (Boulton, 2002; Ramsell et al., 2009). The MP acts in the cell-to-cell movement and CP encapsidates the V-sense ssDNA and acts as a nuclear shuttle protein for viral DNA. CP seems to regulate the balance between ssDNA and dsDNA accumulation (Brown et al., 2012). CP is also needed for virus transmission by the vector (Boulton, 2002). The mastrevirus genome also contains two non-coding regions: the large intergenic region (LIR) and the small intergenic region (SIR). The intergenic regions are of importance in the replication stage since in LIR there is a conserved region where a nick is introduced for replication. In SIR a sequence is found that can act as a primer in the early steps of viral replication (Gutierrez, 1999). Rep is the protein that initiates the rolling circle replication by introducing the nick in the conserved TAATATTAC region found in LIR, on the V-sense strand. It is not yet confirmed, but it is

suggested that Rep has a role in the recruitment of host replication factors to the origin of replication, since Rep binds to the large subunit of the replication factor C loader complex. RepA regulates the cell-cycle progression by binding to the plant homologue of retinoblastoma protein. This will provide host factors that support the viral DNA replication by altering the environment of terminally differentiated cells. RepA also binds to the origin of replication (Brown et al., 2012).

Mastreviruses are transmitted by leafhoppers in a persistent non-propagative manner and are mostly found in the Eastern Hemisphere. They can infect cereals and vegetable crops, and many can also infect wild grasses. For new isolates to belong to the same species in the genus *Mastrevirus*, it has so far been considered to be when the nucleotide identity is greater than 75 % (Brown et al., 2012). In a more recent study by Muhire et al. (2013), it was proposed that a mastrevirus isolate with a nucleotide identity greater than 78 % belongs to the same species. If the identity is greater than 94 %, the isolate are considered to be a members or variants of a specific strain (Muhire et al., 2013).

So far, two separate strains of WDV have been described, but not yet accepted (Brown et al., 2012), one that mostly infects wheat and one that mostly infects barley (Köklü et al., 2007). The host range for the two strains overlap in grasses of the family Poaceae (Ramsell et al., 2009). In the recent study by Muhire et al. (2013), it was suggested with the new thresholds for species and strains, that the wheat-infecting and the barley-infecting types are of different strains. The new suggested classification would result in three separate species, WDV, *Wheat dwarf India virus* (WDIV) found in India, and *Oat dwarf virus* (ODV) isolated in Germany on oat. The WDV species would be divided into five different strains, WDV-A, WDV-B, WDV-C, WDV-D, WDV-E, with the wheat isolate from Sweden belonging to the WDV-E strain (Muhire et al., 2013).

Characteristic symptoms of wheat dwarf disease are dwarfing, yellow streaks on leaves, reduced heading and it also might give a drastic reduce in yield (Kvarnheden et al., 2002; Ramsell et al., 2008). In Sweden in 1997 WDV caused about a 80 % loss in wheat yield in single fields (Lindblad, 2000; Manurung et al., 2004). The wheat of interest is bread wheat (*Triticum aestivum*), since bread wheat accounts for 20 % of the calories consumed by humans. Wheat is also an important source for protein, vitamins and minerals. Wheat originated from a hybridization of the tetraploid emmer wheat and the diploid goat grass about 8,000 years ago (Brenchley et al., 2012).

Wheat dwarf disease was first demonstrated to be caused by a virus by Vacke (1961) in former Czechoslovakia when it had come to attention that the leafhopper *P. alienus* was present on plants where wheat dwarf disease had been observed. The suspicion that it was a virus came from previous experiences with virus diseases in cereals. It stood clear that *P. alienus* was the cause of the disease and with the result of the study it was proven that wheat dwarf disease is caused by a virus, the *Wheat dwarf virus* (Vacke, 1961). The wheat dwarf disease was noticed as early as 1902 in the region around lake Mälaren in Sweden (Nilsson-Ehle, 1918). It has also been observed in many other parts of Europe such as Czech Republic (Vacke, 1961) mentioned previously, Hungary (Bisztray et al., 1989), France (Bendahmane et al., 1995), Germany (Huth, 2000; Schubert et al., 2007), Spain (Achon et al., 2006), Bulgaria (Tobias et al., 2009), and Turkey (Köklü et al., 2007), but it has also been observed in Tunisia (Najar et al., 2000), Syria (Ekzayez et al., 2011), China (Wang et al., 2008), Iran (Behjatnia et al., 2011), and Zambia (Kapooria and Ndunguru, 2004).

P. alienus has in Northern Europe two generations per year and it overwinters in the egg stage. The wingless nymphs pass through five instars before they reach the adult stage. The first and primary infection occurs in the autumn when adult leafhoppers migrate to newly sown fields and the females lay eggs on the seedlings. In spring and early summer when the newly hatched nymphs acquire the virus from the plants infected in the autumn, the second spread take place (Lindblad and Arenö, 2002). With warmer winters and autumns the risk of transmission of WDV to winter crops such as winter wheat will increase. With the current climate, winter crops are sown when the number of active leafhoppers has significantly decreased (Roos et al., 2011). In a study made by Lindblad and Arenö (2002) it was observed that the numbers of caught leafhoppers were low, almost none when the temperature was below 10 °C and quite high when the average temperature during the weeks were around 15 °C. The risk of the plants getting damage from the WDV infection is higher when the plants or grasses are infected in the autumn, if not infected the damage is quite limited. When wheat reaches the stage when the first node is detectable it shows an evident resistance for WDV, which is seen in mature plants. This means that when the adult *P. alienus* leafhoppers are ready to transmit the virus between fields, the crops have already reached the stage of resistance (Roos et al., 2011). Something that have also increased the occurrence and favoured the spread of WDV is the change made in the agricultural practice. Winter wheat is sown quite early which increases the likelihood that the leafhoppers carrying the virus will invade fields and spread the disease (Lindblad and Arenö, 2002).

In this study the aim was to analyse leafhopper samples collected in the county Skåne in Sweden 2010 and that had tested positive for WDV. The samples of interest were chosen because 2010 was the first year wheat dwarf disease had been observed in Skåne for almost a hundred years. At that time, the occurrence was only at a small scale (Nilsson-Ehle, 1918; Waern, 2010).

1.2 Objectives

The objective with this thesis was to determine the DNA sequence of WDV isolates from Skåne (one sample from Bjällerup and four from Haglösa) collected in October 2010, and to perform a phylogenetic analysis to see if it was a different genotype or the same genotype as observed in other parts of Sweden. A second objective was to test primers for WDV to amplify the whole genome in a single PCR. When analysing the primers different samples were used.

Based on the previous study by Ramsell et al. (2009) where they performed a whole genome amplification of the barley strain of WDV, the hypothesis was that the whole genome amplification of the wheat strain would be possible. In another study by Ramsell et al. (2008), they studied the genetic diversity in the wheat strain of WDV and saw a sequence diversity around 3 %. The hypothesis was that the sequences of the WDV isolates from Skåne would be very similar to the previously sequenced and analysed isolates from the counties of Östergötland and Uppsala, Sweden (Kvarnheden et al., 2002; Ramsell et al., 2008).

To determine the sequences, PCR with primers for amplifying the complete genome, rolling circle amplification, and cloning vectors were used (Ramsell et al., 2008; Tobias et al., 2009). Sequences were obtained from sequencing of samples, retrieved from GenBank, and from a master thesis performed in the department (Yazdkhasti, 2012). The sequences were analysed with alignment and phylogenetic analysis (Tamura et al., 2011).

2. Material and methods

2.1 Primers

Primers had been designed by Ingrid Eriksson (Table 1) in the conserved RepA (Kvarnheden et al., 2002) from a partial WDV sequence from Skåne, and they were tested to see whether the full-length genome (2750 nt) of WDV could be amplified. The designed primers had the restriction site for *Hind*III (underlined in Table 1). Also the primers Bar12 and Bar13 for amplification of the full-length WDV barley genome were tested (Ramsell et al., 2009). Primers C1 and C1/C2 (Table 1), which amplify the complete RepA, were used as a control.

Table 1. Primer sequences used in the study. *Hind*III restriction site is underlined in the sequence.

Primer	Sequence 5´ to 3´
C1/C2 (f)	ATGGCCTCTTCATCTGCACC
C1 (r)	CTAGAGACCTTGCCCAGGAA
WDV seqv. F (SeqvF)	CACGAAGCTTGTTCTGCACG
WDV seqv. R (SeqvR)	GAACAAGCTTCGTGCTTCCA
WDV seqv. R2 (SeqvR2)	AGAACAAGCTTCGTGCTTCCA
Bar12 (f)	AAGCTTGTTTTGCACGAGTAC
Bar13 (r)	AAGCTTCGTGCTTCCATCACCAAT

2.2 Virus samples and sample preparation

2.2.1 Plant samples

An extract from a WDV-positive wheat plant from the greenhouse, kindly provided by Jim Nygren was diluted 3:1 and 50 µl was distributed to nine PCR tubes, a tenth tube was prepared with 10 mM Tris-HCl buffer (pH 7.5) as a negative control. All tubes were incubated at 4 °C overnight allowing the virus particles to attach to the plastic. The tubes were washed three times with 10 mM Tris-HCl buffer (pH 7.5).

Fresh wheat leaves with visual symptoms of WDV infection were collected in the greenhouse. Disks of the plant material were collected in PCR tubes by holding the leaf over the tube and closing the lid, creating a plant disk. Four disks were collected from the leaves: two samples from the top and bottom of a large leaf, one from the middle of a large leaf, and the fourth sample from the middle of a small leaf. A DNA extraction was done by adding extraction

buffer (Extract-N-Amp™ Plant PCR Kit, Sigma) to the plant material (Shepherd et al., 2008). The samples were vortexed and quickly centrifuged. The extracts were incubated in a MyCycler™ thermal cycler (Bio-Rad) for 10 min at 95 °C. Again the samples were mixed and centrifuged.

2.2.2 Leafhopper samples

DNA was extracted from two leafhopper samples (sample 3 from Brunnby, Uppsala and sample 29 from Kävlinge, Skåne) by transferring 15 µl of homogenate (leafhoppers homogenised in 10 mM Tris-HCl buffer pH 7.5 three years previously and stored at -20 °C) to new tubes and adding 50 µl of extraction buffer (Extract-N-Amp™ Plant PCR Kit, Sigma). The solutions were mixed and heated to 95 °C for 10 min and then cooled down on ice (Shepherd et al., 2008).

Five additional samples of leafhoppers collected in Skåne, Sweden in 2010 (sample 31 from Bjällerup and samples 32-35 from Haglösa) that had been treated as the previous samples, underwent DNA extraction as previously described.

2.3 Rolling circle amplification

The DNA extracted from homogenised leafhoppers and one sample of previously extracted wheat DNA was used for amplification of circular DNA with rolling circle amplification (RCA) using Illustra TempliPhi™ kit (GE Healthcare). Five µl of sample buffer was mixed with 1 µl of extracted DNA, which was denatured at 95 °C for 3 min and cooled down on ice. After adding 5 µl of reaction buffer and 0.2 µl of phi29 DNA polymerase mix the reaction ran for 18 h at 30 °C in a C1000™ Thermal Cycler (Bio-Rad) and it was terminated by incubation for 10 min at 65 °C (Inoue-Nagata et al., 2004). Approximately 2 µl of the products were analysed on a 1 % agarose gel to see if the amplification had been successful. In a reaction volume of 10 µl, 1 µl of the products was digested with *Hind*III or *Sac*I in a mixture of 1x FastDigest buffer (Thermo Scientific) and 0.5 µl of enzyme *Hind*III or *Sac*I. The mixtures were incubated in a 37 °C heat block for approximately 1.5 h. The digested products were analysed on a 1 % agarose gel.

2.4 Polymerase chain reaction

A PCR was done directly in the tubes of the WDV-positive wheat samples incubated overnight (Wyatt and Brown, 1996). The reaction volume was 50 µl with the final concentrations 1X DreamTaq reaction buffer, 0.2 µM for each primer (SeqvF, SeqvR, C1, and C1/C2), 0.2 mM of dNTPs, and DreamTaq polymerase 5 U/µl (Thermo Scientific). Eight tubes got primers SeqvF/SeqvR (for the gradient) and two tubes got primers C1 and C1/C2, one as a control for presence of WDV and one for contamination control. The amplification of the viral DNA was performed in a MyCycler™ Thermal Cycler (Bio-Rad) starting with heating at 95 °C for 2.5 min, followed by 35 cycles of 95 °C for 30 s, and different annealing temperatures (one for each of the eight samples) at: 65 °C, 63.8 °C, 62 °C, 59.1 °C, 55.5 °C, 52.9 °C, 51 °C, 50 °C for 1 min, and 72 °C for 2 min. The last cycle was followed by 10 min of elongation at 72 °C.

One more PCR reaction was performed using the DreamTaq reagents but using only a 20 µl reaction volume and 0.4 µM of primers and an annealing temperature at 57 °C for 1 min.

More PCR reactions were performed using AmpliTaq Gold® (Invitrogen) reagents 1X AmpliTaq Gold Buffer II, 2.5 mM $MgCl_2$, 0.4 µM of primers, and AmpliTaq Gold® DNA Polymerase 5 U/µl (Invitrogen). The PCR program was run at 95 °C for 10 min, followed by 35 cycles of 30 s at 95 °C, 1 min at 55 °C, and 3 min at 72 °C. The last cycle was followed by 10 min of elongation at 72 °C.

The RCA product was used as a template for PCR using AmpliTaq Gold® (Invitrogen) reagents and same concentrations and conditions as previously. The samples with the RCA product as a template were digested with *Hind*III or *Sac*I. All products were analysed on a 1 % agarose gel.

2.5 Cloning and sequencing

2.5.1 The PCR products

From the gel analysis, bands from the gels of the correct size (~2.7 kb) were cut out and the products were purified from the gel pieces using GeneJET™ Gel Extraction Kit (Thermo Scientific) according to the manufacturer's protocol. Binding buffer at a ratio 1:1 was added to the gel pieces and isopropanol was also added in the same amount as the binding buffer, the extraction was performed once more but without isopropanol. The solutions were mixed and

incubated at 53 °C until the gel had melted. All solution was transferred to columns and the DNA was eluted after centrifugations at 13 000 rpm, and addition of washing buffer and elution buffer according to the protocol. The second time the product was eluted with 25 µl elution buffer instead of the recommended 50 µl because of low concentrations.

The concentration of the eluted DNA was measured with a NanoDrop ND-1000 Spectrophotometer (Fisher Scientific) and controlled by running a small amount on a 1 % agarose gel before the DNA was used for ligation into pGEM®-T Easy Vector (Promega) following the manufacturer's protocol. The ligation reaction was incubated at 4 °C overnight.

Four µl of the ligation solution was transformed into *Escherichia coli* DH5α chemically competent cells (Invitrogen) according to the manufacturer's protocol, except for after the heat-shock when the cells were put on ice for 5 min and 950 µl of LB media was added instead of SOC. The DNA ligation and transformation controls were omitted. Two different volumes (50 µl and 200 µl) of bacterial cells were spread on plates containing LB and Ampicillin (Amp) that had been prepared with X-Gal (20 mg/ml) and IPTG (100 mM). The plates were incubated overnight at 37 °C.

The remaining bacterial cells were spread on plates as described above for backup. Colonies from the plates that had been incubated overnight at 37 °C were collected and put in water. Control of presence of insert DNA was done with PCR with a small amount of the cell solution used as template. The PCR was performed with DreamTaq and a reaction volume of 20 µl with 1X DreamTaq reaction buffer, the primer pair M13F/M13R (0.1 µM of each primer), 0.2 mM dNTP, and DreamTaq polymerase (Thermo Scientific). The PCR was run in MyCycler™ thermal cycler (Bio-Rad) with the same program as previously but with only 30 cycles and an annealing temperature at 50 °C. The PCR products were analysed on a 1 % agarose gel.

The rest of the cell solution was added to 4 ml of LB and ampicillin (10 µg/ml) and the solution was incubated on a shaker (218 rpm) at 37 °C overnight. The cells were collected by centrifugation for 2 min at 6000 rpm. The plasmid DNA was extracted with GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's protocol. The concentration of the eluted DNA was measured with NanoDrop technology (Fisher Scientific) and the DNA was diluted to 20 µl with a concentration of 100 ng/µl and sent to Macrogen for sequencing using T7 (F) and SP6 (R) universal primers. The clones were also analysed by digestion with

the restriction enzyme *Eco*RI in the same manner as the previous digestions. The digestions were analysed on a 1 % agarose gel.

Colonies from the backup plates were also picked and treated as described above and sent for sequencing with the same instructions as previously.

2.5.2 The RCA products

The RCA samples digested with *Hind*III or *Sac*I were ligated into pBluescript® II KS +/- that had been digested with the same enzymes and treated with alkaline phosphatase by Ingrid Eriksson at the department. The same protocol for the ligation was followed as previously, but the ligation was incubated at 16 °C overnight. Five µl of ligation solution was transformed into 50 µl DH5α chemically competent cells (Invitrogen) and the cells were spread on LB and Amp plates prepared as previously with IPTG (100 mM) and X-gal (20 mg/ml).

Colonies were transferred to 30 µl of water, and the clones were tested for inserts by PCR. The control was done on the colonies as previously with M13 primers and DreamTaq. The colonies with positive bands were transferred to 4 ml of LB and Amp (10 µM) and incubated on a shaker overnight at 37 °C. For cultures with visible growth, the cells were collected by centrifugation for 2 min at 6000 rpm and the DNA was purified as previously with GeneJET Plasmid Miniprep Kit (Thermo scientific) according to the manufacturer's protocol. The concentration of the purified DNA was measured with NanoDrop technology (Fisher Scientific) and the presence of insert was controlled with PCR using the primers SeqvF/SeqvR2 and AmpliTaq Gold as described previously. However, the thermal cycler program was set to 30 cycles instead of 35 cycles. Also a digestion with *Eco*RI was performed as previously as a control that plasmids with inserts of the expected size had been purified. After analysing the gel images it was decided that the DNA would not be sent for sequencing.

2.4 Sequence analyses

To get more sequence from the clones than the parts retrieved when sequencing with T7 and SP6 universal primers, new primers were designed approximately 100 nt before the location where the sequence could no longer be read: forward primer 5'GCAGATGAAGAGGCCATGGT3', reverse primer 3'GCGAGTCATTCATCAACTAC5'. To ensure accurate alignment of the sequences, all partial sequences were combined to form the longest possible sequences using the Molecular Evolutionary Genetics Analysis software (MEGA) version 5.1 (Tamura et al., 2011). The reverse complementary of all reverse sequences were aligned and combined with the corresponding forward sequences, building

the longest possible WDV sequences. The determined sequences and the sequences retrieved from GenBank the 5th of April 2013 and Yazdkhasti (2012) were analysed using MEGA, and nucleotide Basic Local Alignment Search Tool (Altschul et al., 1990). The sequences were aligned using ClustalW, integrated in MEGA. The phylogenetic tree was constructed using Maximum Likelihood (ML) designed from the Tamura-Nei substitution model, 1000 bootstrap replications and with *Oat dwarf virus* (AM296025.1) as an outgroup.

3. Results

To be able to acquire the genome sequence of WDV from the leafhopper samples primers had to be tested and evaluated to see their effectiveness in amplifying the entire WDV genome.

3.1 Test of primers

The PCR gradient test with the first primers SeqvF/SeqvR and PCR tubes incubated with a WDV-infected wheat plant was negative (Fig. 1). In an additional test when testing primers SeqvF/SeqvR on infected wheat no positive result was obtained. Only a lot of unknown background products were obtained around 0.2 kb that was present for all samples including the control (Fig. 1). After studying the primer sequences it was decided that the reverse primer should be re-designed.

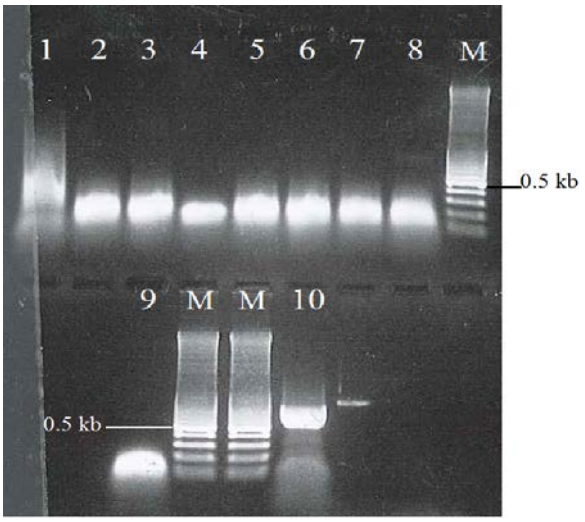

Figure 1. PCR gradient for full-length genome amplification of WDV with SeqvF/SeqvR primers (expected size 2.7 kb). Annealing temperatures used: 1. 60 °C, 2. 63.8 °C, 3. 62 °C, 4. 59.1 °C, 5. 55.5 °C, 6. 52.9 °C, 7. 51 °C, 8. 50 °C, M is MassRuler™ 10 kb ladder mix, 9. Negative buffer control with primers C1 and C1/C2, 10. Positive control with primers C1 and C1/C2 (expected product size 0.75 kb).

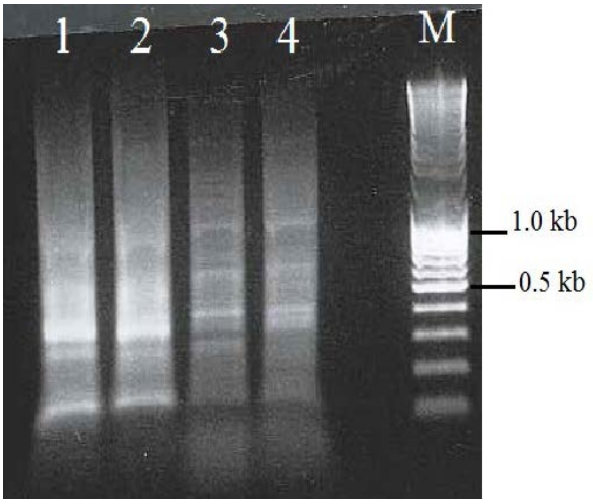

Figure 2. PCR of WDV from infected wheat, testing different primer combinations (expected product size 2.7 kb). 1. SeqvF/SeqvR2, 2. SeqvF/Bar13, 3. Bar12/SeqvR2, 4. Bar12/Bar13, and M is MassRuler™ 10 kb ladder mix.

PCR performed with primers SeqvF/SeqvR2, two DNA samples from infected wheat, and AmpliTaq Gold instead of DreamTaq, still did not yield bands of the right size but only a lot of background of low molecular size. The positive controls with primers C1 and C1/C2 gave a positive result (results not shown).

When testing the different combinations of primers, results that potentially could be positive were obtained with the primer combinations Bar12/SeqvR2 and Bar12/Bar13, but also a lot of smear was obtained (Fig. 2). A band of the correct size for the complete WDV genome would be around 2.7 kb. In Fig. 2 small bands appear at what could be approximately 1.0 kb, which is not correct, but larger products were also produced, which meant that the primers may have worked. With the primers probably working the project proceeded with extracting the viral DNA from leafhoppers and amplifying the DNA.

3.2 DNA extraction from leafhopper samples

No good method for extracting the DNA from the leafhoppers was fully established in the beginning of the project. Previously the virus particles have been retrieved from incubation of WDV-positive samples in plastic tubes (Shepherd et al., 2008). However, since the DNA was going to be used for RCA because the initial PCR amplifications were not as successful as expected, another method was needed. The DNA extraction with the extraction buffer (Extract-N-Amp™ Plant PCR Kit, Sigma) was proven to be successful when studying the gel images for the PCR performed with AmpliTaq Gold® and primers C1 and C1/C2 and DNA extracts from leafhoppers and wheat (Fig. 3). The bands were estimated to be about 0.75 kb, which is the expected size. The successful DNA extractions lead to that the DNA could be amplified with RCA followed by PCR.

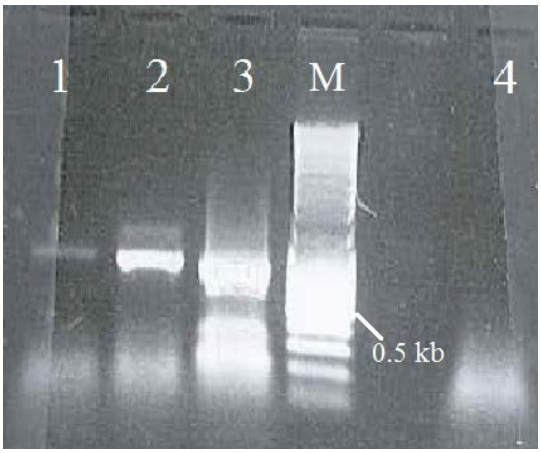

Figure 3. PCR of WDV with DNA from samples of leafhoppers and wheat (expected size 0.75 kb). 1. Leafhoppers from Brunnby (sample 3), 2. Leafhoppers from Kävlinge (sample 29), 3. DNA from infected wheat, M is MassRuler™ 10 kb DNA ladder mix, 4. Negative control with water.

3.3 Rolling circle amplification of leafhopper samples

To be sure that the RCA would work it was first tested in two samples from Brunnby, Uppsala and Kävlinge, Skåne. The first RCA of the leafhopper samples from Brunnby, Uppsala and Kävlinge, Skåne was somewhat successful, with no clear band but more of a smear appearing on the gel (Fig. 4). When the RCA products were digested with *Hind*III or *Sac*I more or less the same type of smear appeared (results not shown). When using the RCA product as a template for PCR, bands of the right size (around 0.75 kb) were obtained. It was obtained for both samples when using primers C1 and C1/C2, indicating that WDV-DNA was present in the samples due to the site-specific primers used in the PCR. Only sample 3 from Brunnby yielded the expected product of 2.7 kb when using primers Bar12/Bar13 and SeqvF/SeqvR2 to get more conformation that it was WDV-DNA that had been amplified (results not shown). The RCA was performed once more but with adding a WDV-infected wheat sample as a positive control. The wheat sample yielded a positive RCA product while for sample 3 (Brunnby) and 29 (Kävlinge) the same kind of smear was visible after gel electrophoresis. Therefore, sample 29 was excluded from further analysis. When digesting the RCA products of sample 3 and the wheat sample with *Hind*III or *Sac*I no specific bands were obtained for sample 3 but for the wheat sample one strong band at 2.7 kb was visible along with two more bands, one high molecular band around 10.0 kb and one smaller around 1.25 kb (results not shown). The extra bands for the wheat sample suggest that DNA from the wheat plant might have been present. The results indicated that it seemed possible that the DNA extraction and RCA worked but some errors still remained. Even though the results were not as satisfying as wished we proceeded with analysing other samples from Skåne since they were the samples of interest in this thesis.

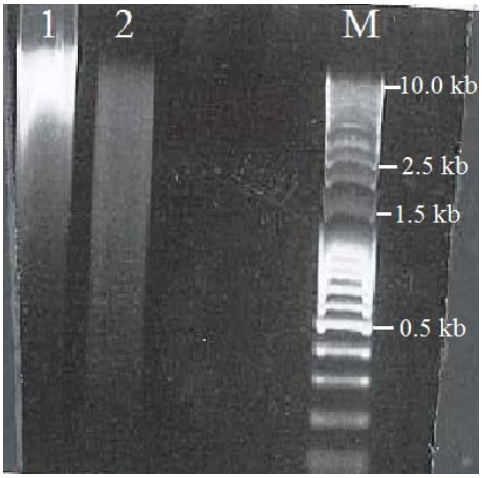

Figure 4. RCA with leafhopper DNA extracts. No clear bands were visible. 1. Brunnby (sample 3), 2. Kävlinge (sample 29), M is MassRuler™ 10 kb DNA ladder mix.

3.4 Rolling circle amplification of samples from Skåne

RCA was performed on the five additional leafhopper samples from Skåne (sample 31 from Bjällerup and samples 32-35 from Haglösa) where the DNA had been extracted as the previous DNA extraction from leafhoppers. RCA product was obtained for all samples but one, sample 31 from Bjällerup (results not shown). PCR with primers C1 and C1/C2, SeqvF/SeqvR2, and Bar12/Bar13 using the positive RCA products as templates resulted in amplification of the expected fragments for samples 34 and 35 with bands at 0.75 kb and 2.7 kb. A faint band was visible for sample 33 at 2.7 kb and a strong band at 0.75 kb that had been amplified with primers Bar12/Bar13 and C1 and C1/C2, respectively (Fig. 5). The RCA products of 32, 33, 34, and 35 were digested with *Hind*III or *Sac*I. This gave again quite good products at 2.7 kb for samples 34 and 35 for both the *Hind*III and *Sac*I digestions, which indicates that it was a WDV genome. A faint band of what seemed to be undigested RCA product was visible for sample 33 and only product from the *Hind*III digested sample 32 at 2.7 kb (results not shown). From the PCR and digestion with restriction enzymes it was decided that samples 32 and 33 should also be excluded from further analysis since it seemed that they may not contain a full-length WDV genome. Sample 33 gave a positive product with primers C1 and C1/C2 but not when using the other two primer pairs and sample 32 was not positive for any of the primer pairs used. The RCA product of sample 32 was only digested by *Hind*III and not *Sac*I, and both restriction sites are usually present in the WDV genome. The analysis continued with purification of the gel products so they could be used for cloning.

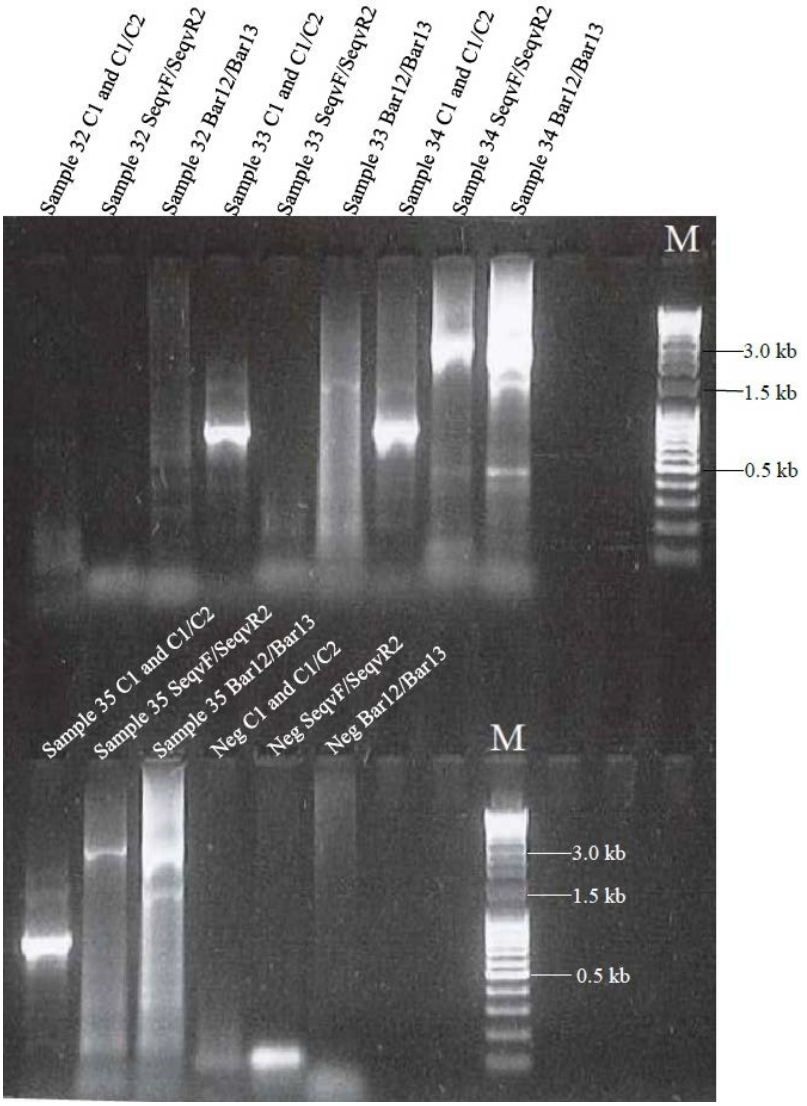

Figure 5. PCR using RCA products as template with primers C1 and C1/C2 (expected size 0.75 kb), SeqvF/SeqvR2, and Bar12/Bar13 (expected size 2.7 kb). The order for all samples are 32 (Bjällerup), 33 (Haglösa), 34 (Haglösa), and 35 (Haglösa) and a negative control with water. All samples were tested with primers C1 and C1/C2, SeqvF/SeqvR2, and Bar12/Bar13. M is MassRuler™ 10 kb DNA ladder mix.

3.5 Ligation and transformation of PCR products from RCA

The purified PCR products were designated as 34 Seqv, 34 Bar, 35 Seqv, and 35 Bar and the concentrations obtained from the NanoDrop ND-1000 Spectrophotometer (Fisher Scientific) measurements can be seen in Table 2.

Table 2. Concentrations from the gel purified PCR products.

Sample	Concentration (ng/µl)
34 Seqv	2.3
34 Bar	5.1
35 Seqv	1.9
35 Bar	1.1

Since the concentrations were quite low, a small amount of the products was run on a gel. The gel image was used to verify that enough DNA had been obtained. The gel image showed bands for all samples (results not shown) and it was decided that the DNA amount was enough to proceed with the ligations. Eight plates were prepared with transformed cells but not many colonies were obtained in the first round. A total of 13 colonies were picked: 2 colonies for 34 Seqv, 8 for 34 Bar, 1 for 35 Seqv, and 2 for 35 Bar. The PCR of the colonies revealed that only eight of the clones seemed to have the correct insert. Two of the 34 Bar clones, the 35 Seqv clone, and both 35 Bar clones were excluded. Digestion with *Eco*RI of the plasmid DNA revealed that the selected clones had the expected insert size of approximately 2.7 kb, yielding bands of 2.4 kb and 0.3 kb, and the vector at 3.0 kb (Fig. 6A).

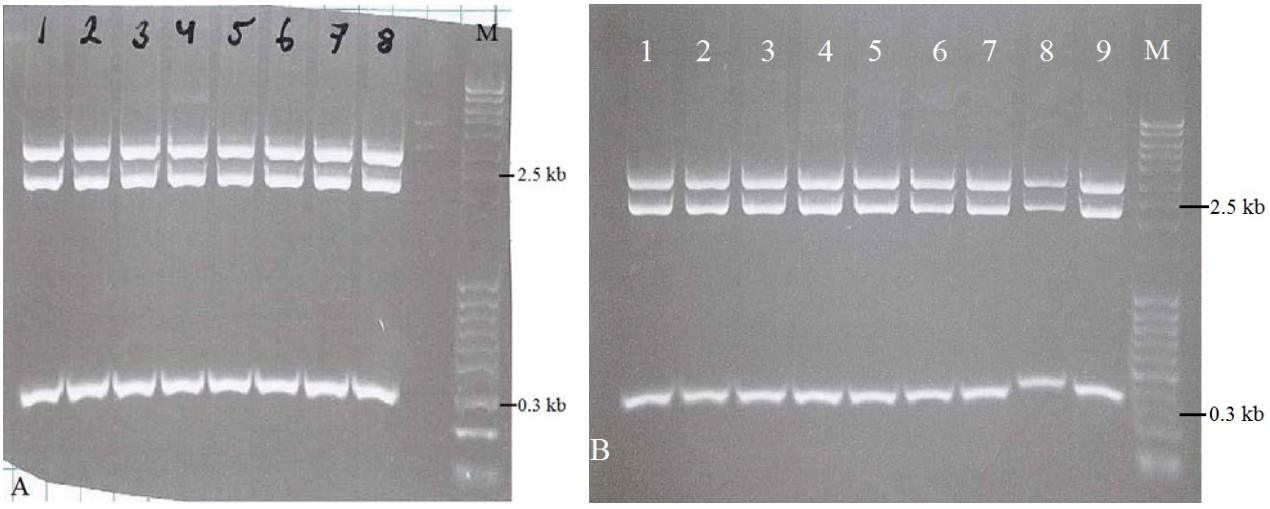

Figure 6. Digestion of the purified plasmid DNA with *Eco*RI yielding the product sizes 3.0 kb, 2.4 kb, and 0.3 kb. A. 1. 34 seqv1, 2. 34 Seqv2 3. 34 Bar1, 4. 34 Bar2, 5. 34 Bar3, 6. 34 Bar4, 7. 34 Bar5, 8. 34 Bar8 M is MassRuler™ 10 kb DNA ladder mix. B. 1. 34 Seqv3, 2. 34 Seqv4, 3. 34 Bar9, 4. 34 Bar10, 5. 34 Bar11, 6. 34 Bar12, 7. 34 Bar13, 8. 35 Seqv2, 9. 35 Seqv3, M is MassRuler™ 10 kb DNA ladder mix.

From the plates made with the remaining cells, 2 colonies were picked for 34 Seqv, 5 for 34 Bar, 2 for 35 Seqv, and 1 for 35 Bar. From the PCR it was decided that the clone for 35 Bar (35 Bar3) should be excluded due to the lack of insert DNA of the right size. The clones kept for further analysis were designated as 34 Seqv3-4, 34 Bar9-13, 35 Seqv 2-3, a total of 9 clones, the concentrations obtained after the extraction is presented in Table 3. The digestion with *Eco*RI gave the same kind of bands as the previous analysis with *Eco*RI with bands at 3.0 kb, 2.4 kb, and 0.3 kb indicating that the insert consisted of the complete WDV genome (Fig. 6B). Because some faults may occur during PCR and that we had received a pBluescript® vector so that the RCA products digested with *Hind*III or *Sac*I could be cloned directly without PCR amplification and ligation into a PCR vector.

Table 3. Concentrations from the purified clonal DNA

Samples	Concentration (ng/µl)
34 Seqv3	505
34 Seqv4	423
34 Bar9	463
34 Bar10	484
34 Bar11	490
34 Bar12	392
34 Bar13	416
35 Seqv2	129
35 Seqv3	429

3.6 Ligation and transformation of *Hind*III and *Sac*I products from RCA

The transformation to clone RCA products gave a lot of colonies with no insert; only small colonies that more seemed to be satellites were visibly white. Twenty-four colonies were picked and were analysed with PCR, 3 colonies were picked for sample 34 digested with *Hind*III, 11 for 34 *Sac*I, and 10 for 35 *Sac*I. No colonies were visible on the 35 *Hind*III plate. From the control PCR with the M13 primers only six clones, 35 SacI 3-8, showed some sign of presence of insert DNA. The sizes were not really the right one at 2.7 kb, the size was almost 2.0 kb, but it was a possibility that some of the clones would later show to be the right ones.

After the bacterial cells had been incubated in liquid media only one clone showed signs of growth, clone 35 SacI 3. The purified plasmid DNA had a concentration of 75 ng/µl, and the digestion with *Eco*RI and PCR using primers SeqvF/SeqvR2 revealed that the insert DNA could not be from WDV since the digestion did not match the previous digestions where three bands had been visible and now only one was visible at approximately 4.0 kb. The PCR gave two faint bands at 10.0 kb and 2.0 kb which is the wrong size for WDV.

Only samples obtained from PCR amplification was sent for sequencing and analysed in MEGA 5.1.

3.7 Sequence analyses

One sequence, 34 Seqv4 sequenced with T7 could not be used due to poor signal in the chromatogram. Clones for samples 34 and 35 were sequenced, with three clones for sample 34 and two clones for sample 35. The results of the sequence reaction were combined to produce contigs of approximately 2.1 kb for each clone. The sequenced clones were all positive for WDV when analysing them in BLASTn, the nucleotide identities to other WDV isolates from the wheat strain were 99 %, which included isolates both from Sweden and isolates from other parts of Europe including Hungary, Germany, and Czech Republic. The highest sequence identity was found to isolates in the WDV-E group suggested by Muhire et al. (2013).

A phylogenetic tree with the new sequences and with the sequences retrieved from GenBank and Yazdkhasti (2012) was constructed including only regions available in the new sequences (Fig. 7). The new sequenced WDV sequences clustered with those determined by Yazdkhasti (2012; SWE WDV 13-1, 13-2, 13-4, 16-1, 16-2, 16-3, 16-4), and with all the WDV-E strain isolates Muhire et al. (2013). Sequences for the Swedish isolates Enk1, 13 and 16 clustered with a bootstrap value of 99 % (Fig. 7), suggesting a close relationship. Another cluster (bootstrap value 70) consisted of the WDV sequences for the two isolates from leafhoppers in Skåne and for other isolates from Hungary, Germany, Czech Republic, and Ukraine (Fig. 7), suggesting that the isolates from Skåne are more closely related to isolates from other parts of Europe than to isolates from central Sweden.

Almost the whole tree structure corresponds to the new classification system suggested by Muhire et al. (2013) where the barley isolates from Bulgaria, Czech Republic, Germany, Hungary, Turkey, and Ukraine belong to strain WDV-A. The barley isolate from Iran belongs to strain WDV-B. Wheat isolates from China, Hungary, and Tibet belong to strain WDV-C. A

wheat isolate found on barley from Iran belongs to strain WDV-D and the remaining wheat isolates from China, Czech Republic, Hungary, France, Germany, Iran, Sweden, and Ukraine belong to strain WDV-E. In the tree (Fig. 7), the WDV-D strain from Iran clusters with the WDV-C strain, which is the only thing that is different from the tree in Muhire et al. (2013).

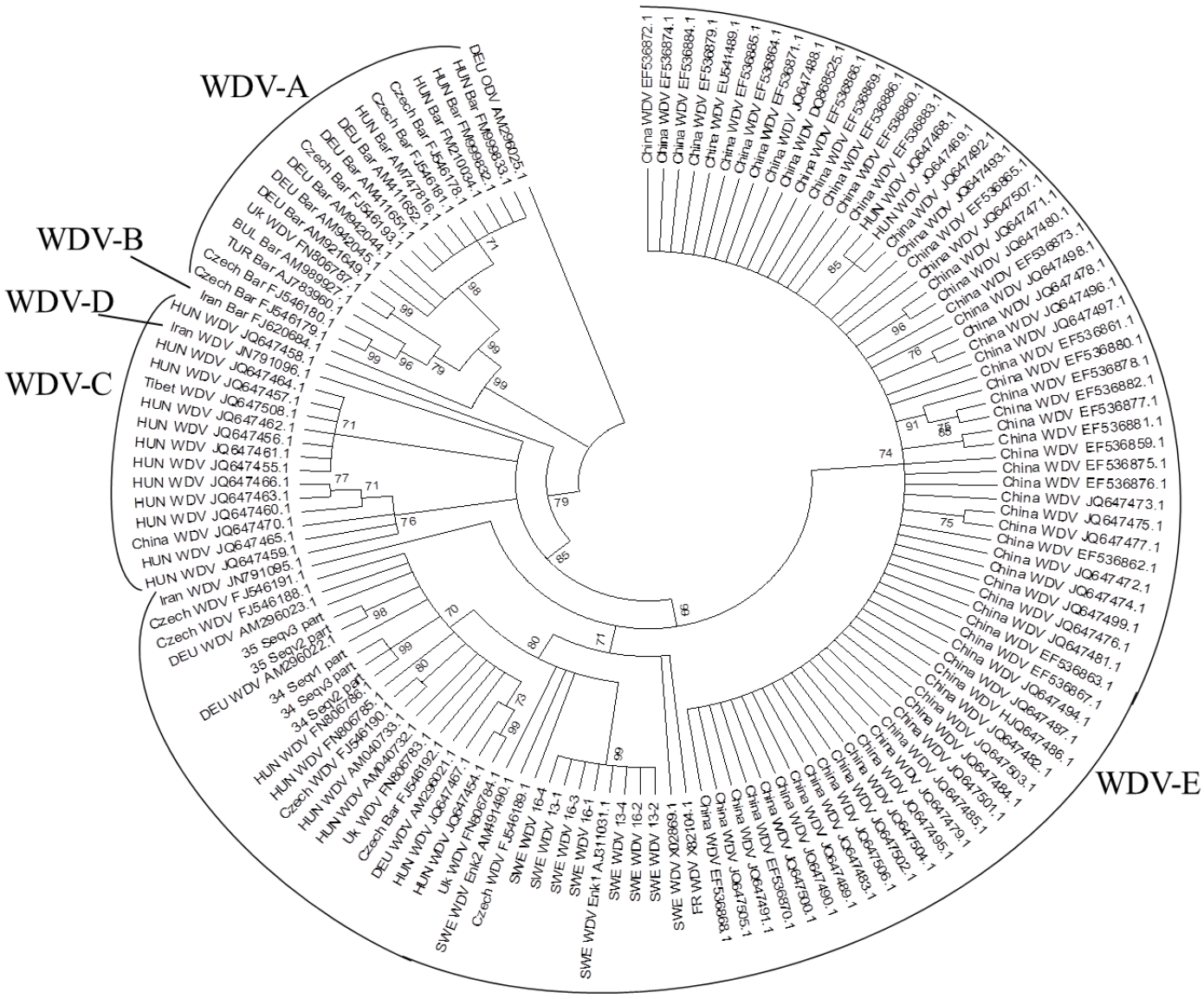

Figure 7. Maximum Likelihood tree constructed using the Tamura-Nei substitution model and 1000 bootstrap replicates with 70 % condensed branches. Strain separation is based on the classification system by Muhire et al. (2013) with *Oat dwarf virus* as an outgroup. The clones sequenced are designated as 34 Seqv1, 34 Seqv2, 34 Seqv3, 35 Seqv3, and 35 Seqv4. Explanation of isolate origins: BUL – Bulgaria, Czech – Czech Republic, DEU – Germany, Fr- France, HUN – Hungary, SWE- Sweden, TUR – Turkey, Uk – Ukraine.

4. Discussion and conclusions

The hypothesis was that the WDV-genome from Skåne would be very similar to the full-length sequences from the counties of Uppsala and Östergötland, Sweden. When analysing the determined sequences in BLASTn the nucleotide identity was 99 % to isolates from Sweden, Germany, Hungary, Ukraine, and Czech Republic. In the phylogenetic analysis, the sequences of the two WDV isolates from Skåne grouped with those of the WDV-E strain. All isolates in the WDV-E strain were found to be closely related and that they did not group according to geographic origins, for example the Swedish isolates did not all group together. The Skåne isolates were positioned in a clade within the WDV-E strain that contained isolates from other parts of Europe, but without any other Swedish isolates. The conclusion that could be drawn is that the isolates from Skåne belong to the WDV-E strain and it is not a new genotype. Because of the high sequence identities to other WDV-E isolates, the Skåne isolates do not seem to be of recombinant origin. When performing the alignment with the partial sequences from the different clones, all full-length sequences retrieved from GenBank and the sequences from Yazdkhasti (2012), the sequences made a good alignment. There are almost 600 nt missing in the partial sequences from a full-genome sequence, and the parts missing are a small part of MP and all of the LIR. Even if LIR has not yet been sequenced, this region is not so conserved and it is not likely that the inclusion of the missing region would affect the result so that it would look like a different strain, or a variant of the wheat strain. Since the WDV genome so far does not seem to have recombined at a large scale (Ramsell et al., 2009), it is not likely that the appearance of WDV in Skåne would be the remit of the emergence of a new genotype.

When wheat dwarf disease appeared in Skåne in 2010, the disease had become visible in a region where it had not been reported since 1915 (Nilsson-Ehle, 1918), and not even in 1915 did the wheat dwarf disease cause problems in Skåne. The reason that Skåne and Denmark have been spared from WDV infections during many years (Waern, 2010) could just be that they have not had the same spread or occurrence of the virus as other parts of Sweden or other countries. It is possible that when walking along the fields you might have observed infected plants at the edges if you had known what to look for. If they have not had a problem with wheat dwarf disease it could be because of limited spread of the virus by leafhoppers. In spring, when wheat is susceptible to WDV infection, the leafhoppers are wingless nymphs and not really able to spread the virus long distances. When mature winged leafhoppers appear wheat has reached a growth stage where it displays mature plant resistance against

WDV (Roos et al., 2011). The reason why leafhoppers positive for WDV were found in the fields in October could be due to the temperature. Temperature measurements in the area around Malmö in September and October 2010 showed that the temperature in October had been over 10 °C at its highest and at an average just below 10 °C, but over 10 °C until the end of September (SMHI, 2013). In the study by Lindblad and Arenö (2002), they observed a higher activity of *P. alienus* when the temperature was above 10 °C. If the temperature had been lower maybe the number of caught leafhoppers would have been reduced.

A lot of problems were encountered in the beginning of the project with absence of amplification when using the primers designed to amplify the entire genome. A lot of background amplification was obtained, which was thought to be the result of the tubes used but this was recognised not to be the case when other tubes were tried. It did not seem to be bad reagents either but the background was reduced when DreamTaq was exchanged for AmpliTaq Gold. The background could also have been caused by DNA extracted from the plants even if it should not be possible since the gradient PCR was run directly in the tubes that had been incubated with extracts of WDV-infected wheat and then washed. Still it is a bit of a mystery what the background was but it more or less vanished as the study proceeded.

At the start of the project, it was not known how to extract viral DNA from leafhoppers to use as a template for RCA. The method by Shepherd et al. (2008) was then tested where originally plant DNA extraction buffer was used in a simple way to extract DNA from plants. When the same method was applied on the homogenised leafhoppers and the extracted DNA was used for RCA it was confirmed to be successful. When the RCA product was used as a template for PCR the gel electrophoresis showed more than one band, which indicates that more than one type of DNA was present, the extra bands were most pronounced when using primers Bar12/Bar13. It could be DNA from the leafhoppers or mutated WDV-DNA with a lower molecular weight, but the extra bands were excluded by gel purification since only the full-length genome was of interest.

The lack of bacterial clones with the right insert for some cloning could be because a protein from WDV was expressed that is toxic for the bacteria. It has been shown that the C2 protein of *Cotton leaf curl Multan virus* (CLCuMV) is likely to be toxic for *E. coli* (Amin et al., 2011). Other reasons why the bacteria did not grow may exist, which means that the problem needs to be further analysed and looked into.

It was unfortunate that no sequences could be obtained for the isolates with amplification by rolling circle since the Φ29 DNA polymerase has a high proofreading capacity (Garmendia et al., 1992). Why amplification by RCA was of interest is also because of possible problems with PCR. PCR was on the other hand not the only method used affecting the final result. Problems that could occur that are directly linked to PCR are the primers and the polymerase. In this study new primers were designed and used as well as previously published ones that had been used for barley isolates of WDV (Ramsell et al., 2009). The previously published primers for barley isolates had a mismatch in the sequence compared to the published wheat isolate Enk1 (Kvarnheden et al., 2002). The new primer pair SeqvF/SeqvR2 for WDV wheat isolates that was designed from a partial sequence from Skåne could also have had a mismatch in the sequence causing problems with the PCR amplification. However, since primers SeqvF/SeqvR2 were designed for a highly conserved region it was not likely that they would target a genome region where mutations or recombination had occurred in the isolates. The polymerase used for PCR typically inserts about one substitution per 10^5-10^6 bases (Schloss et al. 2011), which could result in a sequence that is not identical with the "real one" in the isolate. Something that also could be considered are the errors introduced during the sequencing, which on the other hand is not only specific for samples amplified by PCR. All the problems found in PCR, design of primers, conditions used in the thermal cycler, but also the method for DNA extraction and purification, all affect the final sequence received from sequencing (Schloss et al., 2011). The errors make the samples amplified by PCR not the best for sequencing when the sequence is used for sequence analysis such as recombination analysis.

In this case it seems like that the PCR went well because when comparing the determined sequences with sequences from GenBank the identities were 99 % when performing BLASTn. Even if the identity is that high recombination could still have occurred in the parts not yet sequenced, even though in two of the clones only approximately 350 nt are missing, and most of those nucleotides are found in LIR. The full genome sequence could have been obtained if not the sequencing over LIR would have been so problematic. For both forward primers used to sequence the WDV genome, the sequence reaction failed from where the hairpin is formed for replication. Why these sequencing problems have arisen is not clear, but with only a partial sequence a recombination analysis has not been done. Even if the BLASTn shows a 99 % identity some recombination could have occurred that might have been picked up in a recombination analysis. Because the identity is so high for more than 2 kb we can

draw the conclusion that no recombination has occurred with a more distant relative but we do not know if it has occurred between closely related isolates.

More work with the sequence of the WDV isolates from Skåne is still needed, since parts of the sequence at present are missing. Not much is missing but it is needed to complete the analysis of the genome. Also more work could be done to understand why the cloning of the digested RCA samples did not go as planned. Was it because of toxicity or was some more easily made mistake done? Something to bring on from this project is that we found primers that did amplify the complete WDV genome as far as could be told from the gel images and the part that has been sequenced. The primers are a success to make it even easier to identify if WDV is present in a sample collected from the fields. Also an easy DNA extraction method used on plants was found to work splendidly for extracting viral DNA from leafhoppers and using it for RCA and PCR. Based on the phylogenetic tree it is possible to say that the isolates within the WDV-E strain are closely related and that we did not find a new genotype of WDV in the samples.

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Department of Plant Biology
Uppsala Biocenter, SLU
BOX 7080
75007 Uppsala, Sweden