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# Analyses of circular DNA molecules in the leafhopper *Psammotettix alienus* for identification of viruses and plasmids

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## POPULAR SCIENCE

Microorganisms are a robust group of organisms that may cause different diseases in plants and animals. Vector is an organism that is capable of transmitting a microorganism that causes disease from one host to another. A single vector may carry different species of microorganisms, it may specifically be fungi, bacteria and viruses. In Sweden, these microorganisms have affected the production of wheat crops at different time points. For example, in 1918, the dwarf disease was reported to cause severe yield losses in wheat in central Sweden. Also, in 2017 there was an outbreak of the wheat dwarf disease with the occurrence of leafhoppers in different wheat fields in Sweden. In Sweden, the dwarf disease in wheat is known to be caused by wheat dwarf virus (WDV), which has been demonstrated to be transmitted by plant-feeding insects, leafhoppers. In this study, we focused on analyses of circular DNA molecules in the leafhopper *Psammotettix alienus* for identification of viruses and bacterial plasmids. WDV was detected in DNA extracts of different leafhoppers. In DNA sequence analyses, the identified WDV isolates were found to be closely related to the isolates of WDV previously reported from Sweden. Also, we characterized small circular DNA molecules of other microorganisms present in the leafhoppers. Interestingly, we identified a DNA molecule associated with bacteria known as wheat blue dwarf phytoplasma (WBDP). WBDP has been shown to cause blue dwarf disease in wheat and can also be transmitted by insect vectors. For example, WBDP has been reported in China to cause severe yield losses in wheat at different occasions. In Sweden, a similar disease has been reported to affect oat and barley in past years. In 1970, it was suspected that bacteria termed as mycoplasma-like organisms were the agent of the disease. To our understanding, this will be the first report to show the detection of wheat blue dwarf phytoplasma in the leafhopper *P. alienus* in Sweden. In addition, we identified circular DNA of a virus known as sphinx, which has been reported to be similar to viruses infecting the bacterium *Acinetobacter*. In previous studies, *Acinetobacter* species have been identified from Arctic freshwater, soil and aphids. Therefore, the data are indicating that these bacteria are common in the environments and can be acquired by insects through feeding on different plants. Generally, this project demonstrated that a molecular method known as rolling circle amplification (RCA) is able to assist in detecting a range of viruses and plasmids.

## ABSTRACT

Microorganisms often depend on vectors for transmission. A single vector may carry different species of microorganisms such as fungi, bacteria and viruses. In Sweden, leafhoppers of the species *Psammotettix alienus* are known as a vector of wheat dwarf virus, but they may be carriers of other microorganisms as well. In this study, we analysed small circular DNA molecules in leafhopper DNA extracts for identification of viruses and plasmids. Using PCR, WDV was detected in different samples of *P. alienus* collected in autumn 2017 in different parts of Sweden. Using blast nucleotide analyses, sequences from cloned PCR products were found to share 99% nucleotide identity to previously sequenced WDV isolates from Sweden. Phylogenetic analysis of a genome region covering part of the replication-associated protein A gene (repA), movement protein gene (MP) and long intergenic region (LIR) (1162 nucleotides) showed that the obtained WDV isolates were closely related to the isolates previously reported from Sweden. Also, rolling circle amplification (RCA) was employed as a means of obtaining the full-length plasmids and genomes of viruses with circular DNA. However, we did not get WDV DNA by the RCA method, but circular DNA of a bacteriophage as well as plasmids of phytoplasma and bacteria, were identified. One cloned RCA product shared 83% nucleotide identity with plasmids of wheat blue dwarf phytoplasma (WBDP). A phylogenetic analysis of 1125 bp covering part of the replication-associated protein gene and non-coding DNA confirmed that the analysed sequence was closely related to WBDP plasmids reported from China. WBDP has been reported in China to cause wheat blue dwarf disease resulting in severe yield losses in wheat at different occasions. A similar disease has been reported in Sweden in past years, whereas, through transmission tests, symptoms were then observed on crop plants, such as rye, oat and barley. In addition, we identified circular DNA of a bacteriophage known as sphinx, which is reported to be similar to phages infecting bacteria of the genus *Acinetobacter*. Generally, the results of this study show that RCA is able to assist in detecting a range of viruses and plasmids.

## Table of Contents

POPULAR SCIENCE .....	i
ABSTRACT .....	ii
LIST OF TABLES .....	iv
ABBREVIATIONS .....	v
1. Introduction.....	1
1.1. Microorganisms.....	1
1.2. <i>Wheat dwarf virus</i> .....	1
1.3. Insect vector - leafhopper <i>Psammotettix alienus</i> .....	2
1.4. Amplification of circular DNA by Rolling Circle Amplification (RCA).....	3
2. Aims and objectives .....	4
3. Material and Methods .....	4
3.1. Insect vector (Leafhoppers).....	4
3.2. Polymerase Chain Reaction (PCR) .....	5
3.4. Cloning and sequencing.....	5
3.4.1. Cloning of PCR products .....	5
3.4.2. Cloning of RCA products.....	6
3.5. Sequence analyses .....	7
3.6. Detection of wheat blue dwarf phytoplasma using PCR .....	8
4. Results .....	8
4.1. Detection of WDV in leafhoppers using PCR.....	8
4.1.1. Restriction enzyme analysis of plasmid DNA .....	9
4.2. Cloned DNA sequences.....	10
4.3. Amplification of circular DNA from leafhoppers using RCA.....	12
4.3.1. Restriction enzyme analysis of RCA products .....	13
4.4. Sequence analyses of cloned RCA products .....	14
4.5. PCR developed to detect plasmid of wheat blue dwarf phytoplasma .....	18
5. Discussion.....	18
5.1. Identification of WDV in leafhoppers .....	18
5.2. Identification of viruses using RCA .....	19
5.3. Identification of plasmids using RCA .....	20
6. Conclusion .....	21
ACKNOWLEDGMENT .....	22
REFERENCES.....	23

## **LIST OF TABLES**

Table 1: Primers used for obtaining complete sequence of WBDP plasmid.....	8
Table 2: Description of WDV isolates from GenBank used for phylogenetic analysis .....	10
Table 3: The putative proteins encoded by the identified WBDP plasmid.....	15
Table 4: ORFs and putative proteins in the complete sequence of the WBDP plasmid.....	15
Table 5: Description of phytoplasmal plasmids used for phylogenetic analysis .....	16

## **ABBREVIATIONS**

LB	Luria Broth
MLO	Mycoplasma-like organisms
ODV	Oat dwarf virus
ORF	Open reading frame
PCR	Polymerase chain reaction
RCA	Rolling circle amplification
ssDNA	Single-stranded DNA
WBDD	Wheat blue dwarf disease
WBDP	Wheat blue dwarf phytoplasma
WDD	Wheat dwarf disease
WDV	Wheat dwarf virus
WRDD	Wheat red dwarf disease



## **1. Introduction**

### **1.1. Microorganisms**

Microorganisms have been widely studied and characterized into different species. They are categorized into two major groups, beneficial and disease-causing microbes (Schirawski & Perlin, 2018). Disease-causing microorganisms together with their vectors have been important topics for molecular pathology studies. For example, in Sweden the study of *Wheat dwarf virus* and its vector leafhopper have been made as effort of combating the problem of wheat dwarf disease (Kvarnheden *et al.*, 2002). Diseases in plants may be caused by a single species or complex community of microbes (Lamichhane & Venturi, 2015). Identification of these microbes can be achieved using different molecular methods. For example, the method of rolling circle amplification (RCA) has been widely used in identification of microorganisms with circular DNA such as viruses and plasmids. Among these microorganisms, viruses have been reported to cause different diseases in plants. The diseases caused by viruses are difficult to be controlled, therefore, the use of disease resistant plants is important for sustainable crop production.

### **1.2. Wheat dwarf virus**

Wheat dwarf virus (WDV) is an economically important pathogen of wheat. Following transmission experiments, it was shown by Vacke (1961) as a causative agent of wheat dwarf disease (WDD), also, molecular studies by Woolston *et al.* (1988) confirmed that WDD is caused by WDV. However, there were incidences of a disease with comparable symptoms to WDD in Sweden already over 100 years ago, but the cause of the disease was then not clearly understood (Kvarnheden *et al.*, 2002).

The species *Wheat dwarf virus* belongs to the family *Geminiviridae* and genus *Mastrevirus*. The viruses of this species have a monopartite genome of circular single-stranded DNA (ssDNA) (Lindsten *et al.*, 1980; Muhire *et al.*, 2013). The genome component of WDV and other mastreviruses has a conserved arrangement of genes encoding two replication-associated proteins, a movement protein and a coat protein (Muhire *et al.*, 2013). These genes are important for regulating viral activity during infection, for example, replication in the host cell, cell-to-cell movement within the host and encapsidation of the virus. Like other mastreviruses, WDV replication depends on the host replication machinery through a rolling circle replication system (Rojas *et al.*, 2005). Initially, two strains of WDV were known, a barley-adapted strain and a wheat adapted-strain (Kvarnheden *et al.*, 2002), but to date five strains of WDV have

been identified. WDV-A, WDV-B, and WDV-D are strains that have been identified on barley; WDV-C and WDV-E are strains that have been identified on wheat (Muhire *et al.*, 2013). Most of the WDV isolates identified from wheat in Europe and Asia share high genome sequence identity and form the strain WDV-E while some WDV isolates identified from wheat in China, Hungary and Tibet form the strain WDV-C (Muhire *et al.*, 2013; Nygren, 2016).

WDD is not only a problem in Sweden, but its incidence has been reported periodically from many parts of Europe, Asia and North Africa (Nygren *et al.*, 2015; Kvarnheden *et al.*, 2016). WDV has a wide host-range, ranging from cereal crop species to wild grasses, which makes it possible for the virus to persist in the fields for a long time. The main economically important crops that can be infected by WDV are barley and wheat, and although it can infect other cereals such as rye, oats, and triticale, the disease symptoms are then usually mild (Manurung *et al.*, 2004). WDV can also infect common wild grasses such as those which are adjacent to affected wheat fields, but the symptoms are usually mild or absent (Ramsell *et al.*, 2008). In a field of infected wheat plants, symptoms that are often seen include stunting and reduced seed set as well as yellowing and chlorotic streaks on leaves. Total crop failure may occur when infection appears in young wheat plants, however, infection of mature plants results in limited damage because they have resistance against WDV (Kvarnheden *et al.*, 2016).

### **1.3. Insect vector - leafhopper *Psammotettix alienus***

The spread of WDV depends on vector transmission, with the leafhopper *Psammotettix alienus* as the main vector of WDV (Lindsten *et al.*, 1970; Manurung *et al.*, 2004). Also, other leafhoppers of the genus *Psammotettix* may be vectors of WDV such as *P. provincialis* (Ekzayez *et al.*, 2010). The *P. alienus* individuals can transmit different strains of WDV after feeding on infected wheat and barley plants, as well as the mastrevirus oat dwarf virus (ODV) (Schubert *et al.*, 2007). Both nymphs and adult leafhoppers can carry virus inoculum and transfer WDV from diseased to healthy plants. In autumn, adult leafhoppers transmit the virus to young winter wheat seedlings while in spring virus transmission is carried out by nymphs (Lindblad & Sigvald, 2004). Leafhoppers transmit WDV in a persistent and non-replicative manner (Lindsten *et al.*, 1970). The virus is acquired through the stylet, accumulates in the salivary glands of leafhoppers and can be injected into plant phloem tissue during feeding (Wang *et al.*, 2014b).

The *P. alienus* leafhoppers are commonly occurring in grasslands and arable fields (Lindblad & Arenö, 2002). Volunteer wheat plants and wild grasses are a breeding place for leafhoppers

and serve as a reservoir of WDV (Manurung *et al.*, 2005). The size of the leafhopper population depends on the temperature. A study in central Sweden showed that the number of *P. alienus* leafhoppers increased when the temperature exceeded 15°C (Lindblad & Arenö, 2002). The leafhopper population decreases when the average maximum temperature is less than 10°C (Lindblad & Arenö, 2002; Roos *et al.*, 2011). The warmer condition favours the activity of leafhoppers which can lead to an increased rate of WDV transmission. Understanding the population behaviour of *P. alienus* is crucial for predicting the epidemiology of WDD and the development of effective disease control strategies. The decrease in temperature in late autumn can reduce the activity of leafhoppers, and therefore late planting of winter wheat can reduce the risk of WDV infection (Lindblad & Arenö, 2002). However, due to climate change a period of warmer temperature up to late autumn has been predicted for the Scandinavian region (Roos *et al.*, 2011). This will increase the activity of *P. alienus* and result in increased spread of WDV.

WDV has been widely studied using diseased wheat plants as a virus source, while the genetic diversity of WDV isolates from different geographic areas has been described (Kvarnheden *et al.*, 2002; Muhire *et al.*, 2013). There are also studies describing detection of WDV in the vector *P. alienus* (Ramsell *et al.*, 2008; Zhang *et al.*, 2010; Wang *et al.*, 2014; Kamali *et al.*, 2017). However, genetic diversity studies on WDV isolates identified from leafhoppers are limited.

#### **1.4. Amplification of circular DNA by Rolling Circle Amplification (RCA)**

Before development of the method of rolling circle amplification (RCA), amplification of circular DNA has been done using traditional molecular tools such as polymerase chain reaction (PCR) by use of specific and universal primers. Nowadays, biomedical and biotechnology research involving amplification of circular DNA has been simplified by use of RCA. RCA is a simple method that uses bacteriophage  $\Phi$ 29 DNA polymerase for circular DNA amplification and does not require thermal cycling like in PCR (Inoue-Nagata *et al.*, 2004; Demidov, 2016). The RCA products can be sequenced directly if their DNA concentrations are sufficiently high or can be used for restriction fragment length polymorphism (RFLP) analysis. The use of RCA products for subsequent sequencing has made RCA into a simple, fast, and low-cost method for obtaining results (Demidov, 2016). This method has been successful in the amplification of circular DNA of begomoviruses (Inoue-Nagata *et al.*, 2004) and WDV (Ramsell *et al.*, 2009). In addition, RCA together with RFLP can be used in identification of plasmids and bacteriophages with circular DNA (Demidov, 2016). Therefore, the RCA method was

employed in this study for amplification of circular DNA of WDV and other potential pathogens.

## **2. Aims and objectives**

Wheat is among the ten most economically important crops in the world, but the production is affected by different pathogens including fungi, bacteria and viruses. However, the understanding of disease in relation to a specific pathogen is still challenging because different pathogens may induce similar disease symptoms. Therefore, molecular characterization of the pathogens is important for understanding the relationship between pathogen, host and vector.

The overall aim of this study was to characterize circular DNA molecules of WDV and other microorganisms in samples of the leafhopper *P. alienus* collected in different parts of Sweden in autumn 2017. More specifically I was interested in the following objectives.

- i) To characterize WDV isolates in samples of the leafhopper *P. alienus* and study their genetic diversity. In the year 2017, there was an outbreak of disease in different wheat fields in Sweden with symptoms of wheat dwarf disease. The collected leafhoppers came from regions previously affected by wheat dwarf disease and these leafhoppers are known as a vector of WDV. In a previous study, we detected WDV in different samples of the leafhopper *P. alienus* but sequence characterization of the virus was not done. Also, I was interested in understanding if the identified WDV isolates were related to isolates previously reported from Sweden.
- ii) Identification of other potential pathogens with circular DNA in the leafhopper *P. alienus*. It has been reported that the leafhopper *P. alienus* can be a vector of different plant viruses. Also, insect vectors have been used in different studies for identification of potential pathogens that may cause disease in different crops. This approach has been found to be more useful than the survey of host plants. The identification of different potential pathogens in plants will require the collection of many plant samples, which is tedious and time-consuming.

The data from this study will be important for understanding the genetic diversity of WDV as well as other pathogens with circular DNA genomes that are vectored by the leafhopper *P. alienus*.

## **3. Material and Methods**

### **3.1. Insect vector (Leafhoppers)**

The *P. alienus* samples used in this study had been collected by the Swedish Board of Agriculture in different fields around Sweden in autumn 2017. Samples from pools of leafhoppers were homogenized and DNA was extracted by use of GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) according to manufacturer's instructions. The extracted DNA was used for PCR and RCA analyses.

### **3.2. Polymerase Chain Reaction (PCR)**

To test the presence of WDV, DNA extracts from pools of leafhoppers were analysed by PCR to amplify a region of the virus genome by the use of the specific WDV primers 1877-1896 and 328-309 (Kvarnheden *et al.*, 2002). These primers have been designed to amplify a fragment of 1201 bp corresponding to nucleotides 1877–2750 and 1–328 of the WDV genome (Kvarnheden *et al.*, 2002). Amplification of viral DNA was performed in a C1000 Touch™ Thermal Cycler (BIO-RAD) starting with 120 seconds of heating at 94°C, followed by 34 cycles of 30 seconds at 94°C, 1 minute at 55°C and 2 minutes at 72°C, and a final extension for 10 min at 72°C (Kvarnheden *et al.*, 2002). The PCR fragments were separated by electrophoresis in a 1% agarose gel.

### **3.3. Rolling Circle Amplification (RCA)**

For obtaining multiple copies of DNA of viruses and plasmids, DNA extracts from samples of the leafhoppers were used for amplification of circular DNA with RCA using bacteriophage  $\Phi$ 29 DNA polymerase (Inoue-Nagata *et al.*, 2004). The RCA was done by use of Illustra TempliPhi Amplification Kit (GE Health Care) according to manufacturer's instructions. The RCA products were separated by electrophoresis in a 1% agarose gel.

### **3.4. Cloning and sequencing**

#### **3.4.1. Cloning of PCR products**

The WDV-positive PCR products were selected and used for cloning. After agarose gel electrophoresis analysis, PCR fragments with an approximate size of 1201 bp were cut out and purified from agarose gel pieces using NucleoSpin Gel and PCR Clean-up Kit (Thermo Scientific) according to the manufacturer's protocol. The purified DNA was controlled by running 5  $\mu$ l on a 1 % agarose gel before setting up a ligation reaction. Cloning was done by use of Clone Jet PCR cloning kit (Thermo Scientific) according to the manufacturer's protocol. However, 10  $\mu$ l of purified DNA was ligated into 1  $\mu$ l vector (pJet1.2/blunt) instead of 1  $\mu$ l of purified DNA recommended by the manufacturer's protocol. The ligations were incubated at room temperature for 30 minutes and used directly for transformation. The plasmid DNA was

transformed into *Escherichia coli* DH5 $\alpha$  competent cells (Invitrogen) by adding 5  $\mu$ l of the ligation product to 50  $\mu$ l of bacterial cells. The mixture was incubated on ice for 30 minutes. Bacterial cells were heat shocked at 42°C for 45 seconds in a water bath and chilled on ice for four minutes. Then, 900  $\mu$ l of LB media was added and the transformed bacterial cells were incubated for two hours at 37°C with shaking at 200 rpm. The bacterial solution was centrifuged for 5 minutes at 4400g to pellet the bacterial cells, followed by removing 550  $\mu$ l of the supernatant and resuspending the cells in 400  $\mu$ l solution. Bacterial solutions of 200  $\mu$ l and 100  $\mu$ l were spread on LB plates containing 100  $\mu$ g/ml ampicillin and the plates were incubated at 37°C overnight. Selected colonies were transferred to Falcon tubes (17x100mm) containing 4 ml of LB medium. The bacterial cultures were incubated at 37°C overnight with shaking at 200 rpm.

Plasmid DNA was purified by use of 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). Purified plasmid DNA was digested with BglII restriction enzyme to check if it contained the expected insert size of 1201 bp. Clones for leafhopper samples 46, 60 and 61 containing the correct insert sizes were sent for sequencing in the forward and reverse direction to Macrogen Europe laboratory (Amsterdam, The Netherlands). Leafhopper sample 46 was collected from Kyrketorp in the county of Västra Götaland while leafhopper samples 60 and 61 were collected from Ringarum in the county of Östergötland.

### **3.4.2. Cloning of RCA products**

Restriction fragment length polymorphism (RFLP) analysis of the RCA products was done using HindIII, EcoRI and SacI restriction enzymes in separate reactions. The digestion was done in three replicates for all samples to increase DNA yield. The enzyme HindIII generally has one restriction site in the WDV genome and digestion then yields a 2.7 kb product, which consist of a linear complete virus genome. The digestion products were then separated by electrophoresis in a 1% agarose gel.

The RCA product which was digested with HindIII yielded fragments with sizes of 0.7 kb, 1.4 kb, 1.7 kb and 1.9 kb. The RCA product which was digested with EcoRI yielded a fragment of 5.4 kb and RCA product digested with SacI yielded a fragment of 3 kb. All fragments were cut out from the gel and the DNA purified using NucleoSpin Gel and PCR Clean-up Kit (Thermo

Scientific) according to the manufacturer's protocol. After purification, the DNA was controlled by running 5 µl of purified DNA on a 1 % agarose gel.

The purified DNA fragments were ligated into a pBluescript KS+ vector (Stratagene), which had been digested with the same restriction enzymes (HindIII, EcoRI or SacI) and dephosphorylated using Shrimp Alkaline Phosphatase (SAP) (Thermo Scientific) to prevent re-ligation. The ligation was run in a reaction volume of 20 µl containing 1 µl pBluescript KS+ vector (53.2 ng), 10 µl of purified DNA fragment or MilliQ water as a negative control, 2 µl of 10X T4 DNA ligation buffer, 1 µl of T4 DNA ligase (Fermentas) and 6 µl of MilliQ water. The ligation was incubated at room temperature overnight and the reaction was stopped by incubation at 65°C for 10 minutes.

Plasmid DNA was transformed into *E. coli* DH5α competent cells (Invitrogen) in a similar way as described above for cloning of PCR product (section 3.4.1.)

Purification of plasmid DNA was done by use of 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). Purified plasmid DNA was digested with HindIII, EcoRI or SacI restriction enzymes to check if it contained the expected insert size. Clones containing correct insert sizes were selected and sent for sequencing using M13 universal primers to Macrogen Europe laboratory (Amsterdam, The Netherlands).

### **3.5. Sequence analyses**

The obtained sequences were analyzed using Blast nucleotide (Blastn) and Blast translated nucleotide (Blastx) for searches of GenBank and protein databases, respectively. The sequences with identity to WDV were selected and assembled from two overlapping sequences in each direction using SeqMan Pro (Lasergene package; DNASTar, Inc.). The sequences for the WBDP plasmid were assembled also by use of SeqMan Pro (Lasergene package; DNASTar, Inc.). Primer walking was used to determine the complete sequence of the WBDP plasmid. To extend the WBDP plasmid sequences obtained by use of vector primers (M13 forward and M13 reverse) specific primers were designed. For obtaining the complete sequence (5438 bp) of the WBDP plasmid, six primers were used to extend the sequences in forward and reverse directions (Table 1).

Table 1: Primers used for obtaining complete sequence of WBDP plasmid

Primer name	Sequence	Direction of extension
WBDPP 3	5'- TGCGT TTCGC TTTGT ACTTG - 3'	Forward
WBDPP 2	5'- CGACA CAAGG AATAA ATTGG - 3'	Reverse
WBDPP 4	5'- CTTTG GGGAT TTAGG GGGTT- 3'	Forward
WBDPP 5	5'- CAGTT TTAAG TTGGG GGTTTC- 3'	Reverse
WBDPP 6	5'- TGCCT GCGCT ACTTA TAGTA - 3'	Forward
WBDPP 7	5'- CCGAC TAAAT CCAA ATAGT - 3'	Reverse

The assembled sequences of WDV and WBDP plasmid were analyzed using an open reading frame (ORF) finder tool from National Centre for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/orffinder/>). To study the relationship between different WDV isolates as well as different phytoplasma plasmids, phylogenetic analyses were carried out using the Neighbour-Joining method (Saitou & Nei, 1987) in the MEGA 7 software (Kumar *et al.*, 2016). The sequences obtained from this study and sequences available from GenBank were aligned using the Clustal W method. The alignments were used to calculate the genetic distances with the Kimura two-parameter model.

### 3.6. Detection of wheat blue dwarf phytoplasma using PCR

To test the presence of WBDP plasmid from the same leafhopper samples that were tested for the presence of WDV, new PCR primers were designed from the obtained sequences of the WBDP plasmid. PCR was run using a forward primer with sequence 5'- CGAAA ACCAT CAAGA CCATA -3' and reverse primer with sequence 5'- ACCAC CACAA GATTC TTCTA -3'. These primers were designed to amplify a fragment of 895 bp covering part of the replication-associated protein gene of the WBDP plasmid identified in this study. The amplification of the plasmid DNA was performed in a C1000 Touch™ Thermal Cycler (BIO-RAD) starting with 1 min of heating at 94°C, followed by 34 cycles of 30 seconds at 94°C, 1 min at 50°C and 2 min at 72°C, and a final extension for 10 min at 72°C. The PCR fragments were separated by electrophoresis in a 1% agarose gel.

## 4. Results

### 4.1. Detection of WDV in leafhoppers using PCR

In a previous study, we performed PCR analyses for samples of leafhoppers collected in different regions around Sweden. Out of 51 tested samples of leafhoppers, 30 samples were then negative for WDV and 21 samples were positive. In this study, 10 samples were tested

again by PCR for the purpose of subsequent cloning and sequencing. After analysis of PCR products by agarose gel electrophoresis, fragments with the expected size of 1.2 kb were found for leafhopper samples 32, 46, 60, 61 and 65 (Figure 1). These PCR fragments indicated the presence of WDV in the leafhopper samples. The PCR products of the WDV-positive samples 46, 60 and 61 were further used for cloning and sequencing.

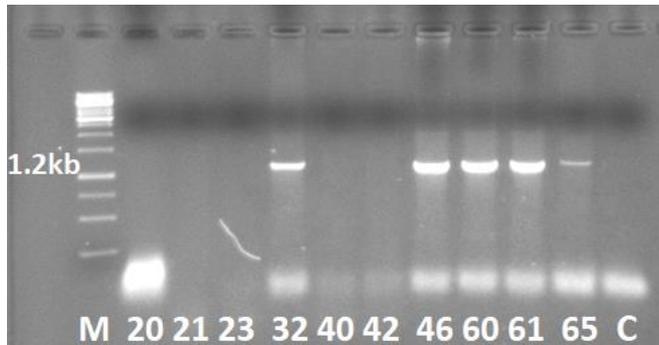


Figure 1: Agarose gel electrophoresis of PCR products. Bands with the size of 1.2 kb for leafhopper samples 32, 46, 60, 61 and 65 are indicating the presence of wheat dwarf virus. Lane M is GeneRuler™ 1kb DNA Ladder (Thermo Scientific). Lane C is a negative control (MilliQ water) for PCR.

#### 4.1.1. Restriction enzyme analysis of plasmid DNA

After liquid culturing of putatively transformed bacterial cells, plasmid DNA was purified. To confirm that the plasmids contained inserts corresponding to the WDV amplification product, they were digested with BglII restriction enzyme. After restriction digest, clones with insert of the correct size of 1.2 kb (Figure 2) were obtained and used for further analysis. One clone for sample 46, four clones for sample 60 (data not shown) and three clones for sample 61 were having insert of the correct size.

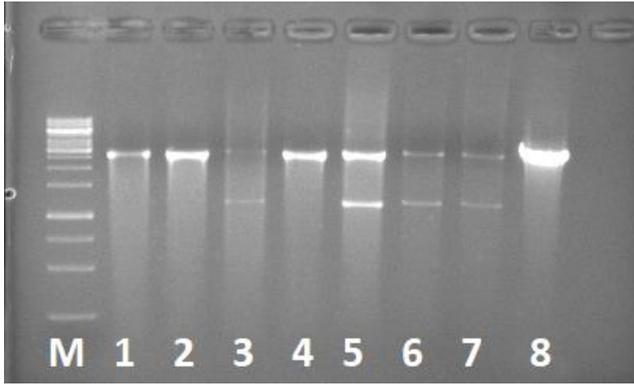


Figure 2: Agarose gel electrophoresis of plasmid DNA digested with BglII restriction enzyme. Lanes 1 to 4 are clones of sample 46 and lanes 5 to 8 are clones of sample 61. Lanes 3, 5, 6, and 7 show correct insert fragments with a size of 1.2 kb corresponding to the WDV amplification product. Lane M is GeneRuler™ 1kb DNA Ladder (Thermo Scientific).

#### 4.2. Cloned DNA sequences

The DNA sequences were analyzed using Blastn and compared with sequences in GenBank. The sequences of six clones (46\_3, 60\_17, 60\_18, 61\_1, 61\_2 and 61\_3) shared 99% nucleotide identity with WDV. However, the complete sequence was not obtained for all clones because of a stem-loop interfering with the sequence reactions. The nucleotide sequence of 1162 nucleotides covering part of the gene encoding replication-associated protein A (repA), movement protein gene (MP) and the long intergenic region (LIR) was used for phylogenetic analysis. In the phylogenetic analysis, other WDV sequences from GenBank were included as indicated in Table 2. Sequences of two barley-infecting WDV isolates were included as an outgroup. The analysed sequence (1162 nucleotides) of WDV isolate 61 (clone 61\_3) was closely related to the isolates previously identified from Sweden (Figure 3). The determined sequences from two clones (60\_17 and 60\_18) of WDV isolate 60 identified in this study were shorter in length (820 bp) and were not included in the analysis in Figure 3. Instead, they were analyzed separately and the results revealed them also to be closely related to WDV isolates from Sweden (Figure 4).

Table 2: Description of WDV isolates from GenBank used for phylogenetic analysis

WDV isolate	Country	Accession number
Enköping1	Sweden	AJ311031
Enköping2	Sweden	AM491490
Haga 5.2	Sweden	AM711135
Ojebro Pp1	Sweden	AM491482
Hacksta	Sweden	AM491475
Froogard 2.1	Sweden	AM491488
Norsholm	Sweden	AM707038
WDV_barley strain	Hungary	AJ311038
WDV_B_barley	Poland	KM079155

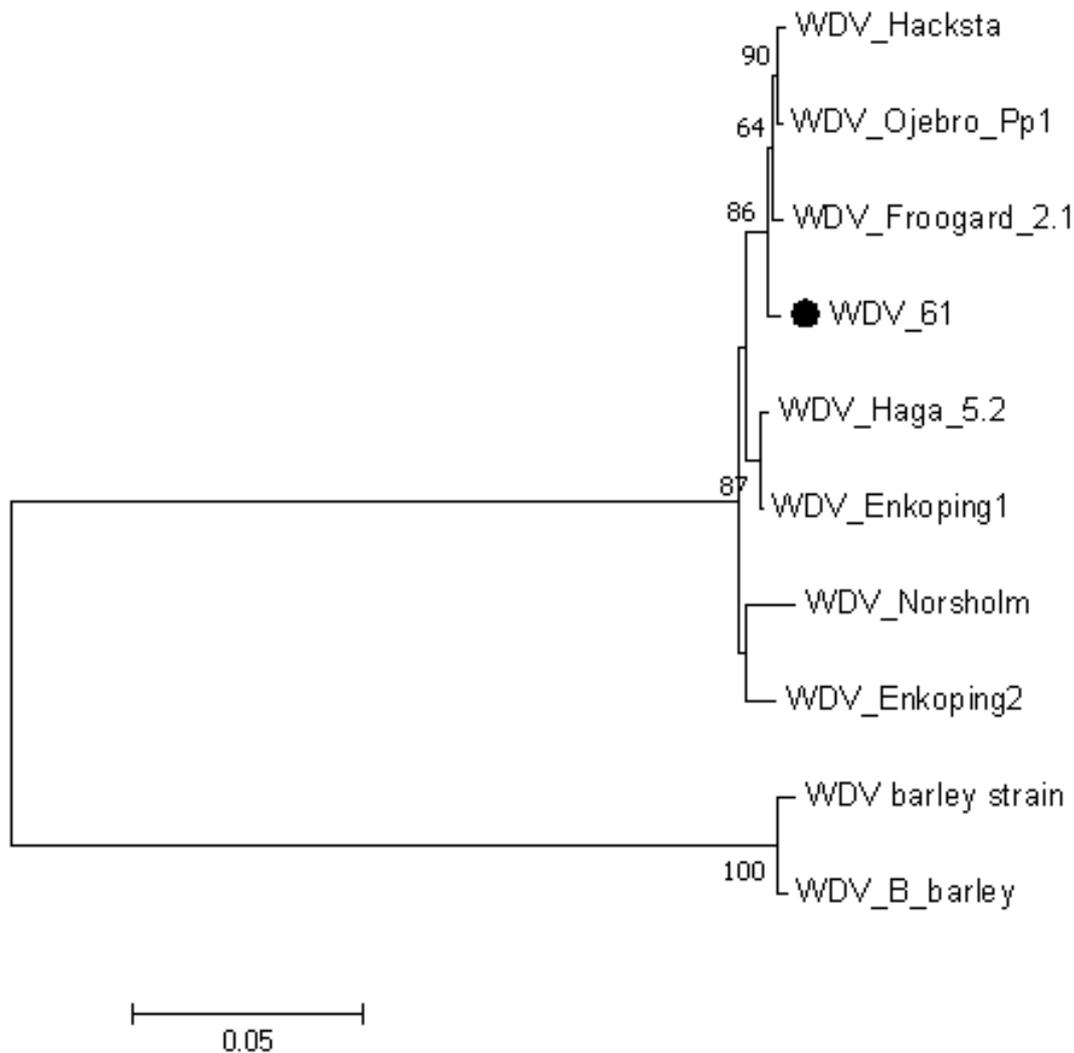


Figure 3: Phylogenetic analysis of 10 WDV isolates based on the alignment of 1162 nucleotides covering LIR and parts of the repA and MP genes. The figure shows a neighbour-joining tree inferred from Kimura two-parameter estimates of nucleotide substitutions per site. The WDV isolate marked with a circle is the one sequenced in this study. Numbers above branches represent the bootstrap values out of 1000 replicates. Only bootstrap values over 60 are shown. Description of virus names and accession numbers are shown in Table 2. Branch length is drawn to scale with the bar indicating 0.05 nt substitutions per site.

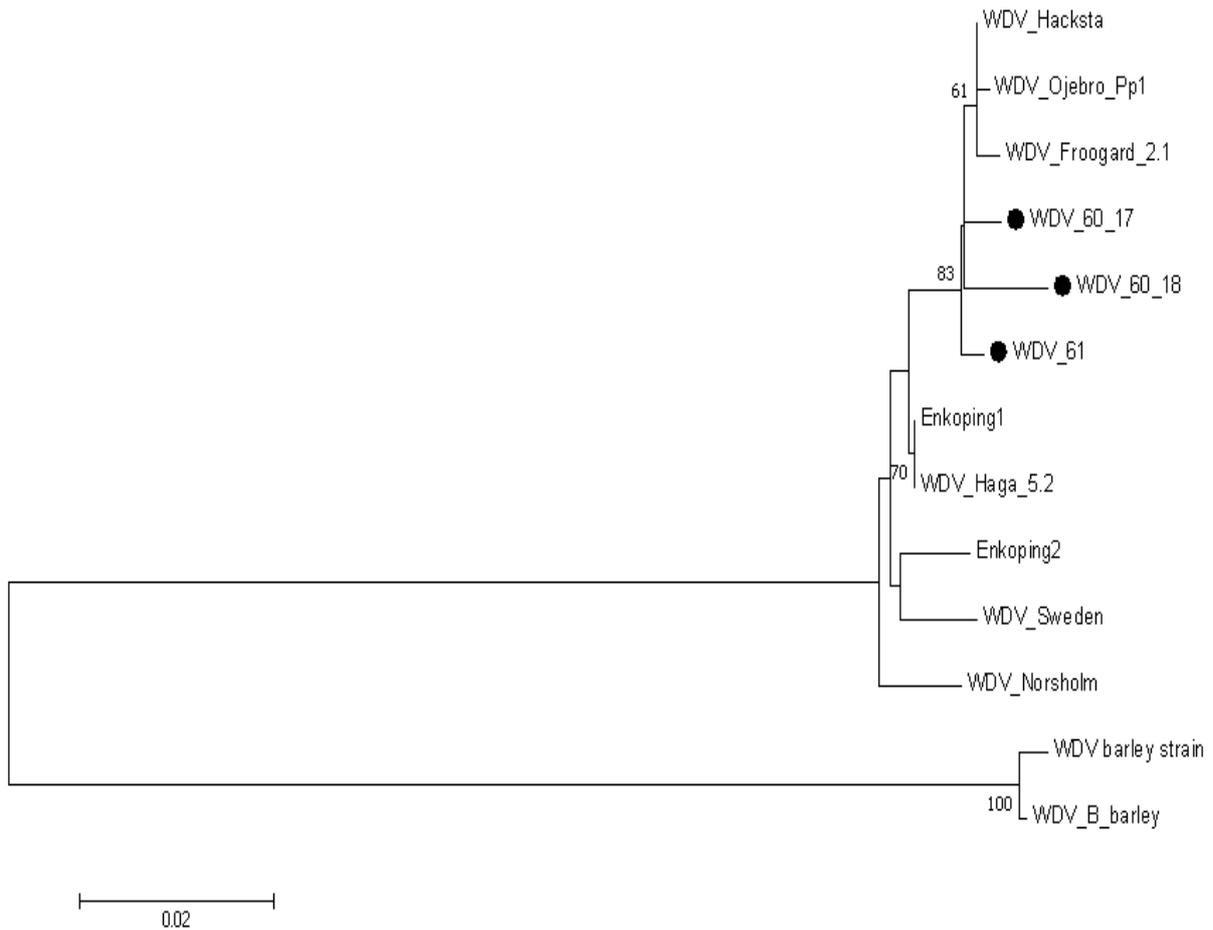


Figure 4: Phylogenetic analysis of 13 WDV isolates based on the alignment of 820 nucleotides covering part of repA gene and LIR. The figure shows a neighbour-joining tree inferred from Kimura two-parameter estimates of nucleotide substitutions per site. The WDV isolates marked with a circle are those sequenced in this study. Numbers above branches represent the bootstrap values out of 1000 replicates. Only bootstrap values over 60 are shown. Description of virus names and accession numbers are shown in Table 2. Branch length is drawn to scale with the bar indicating 0.02 nt substitutions per site.

### 4.3. Amplification of circular DNA from leafhoppers using RCA

The RCA method has been used in different studies for amplification of circular DNA and it has become an important tool for biomedical and biotechnology research. It was also successfully used in this study to generate amplification products from DNA extracts of leafhoppers. Following agarose gel electrophoresis analysis, high-molecular weight bands corresponding to RCA products were visualized for 9 samples (Figure 5).

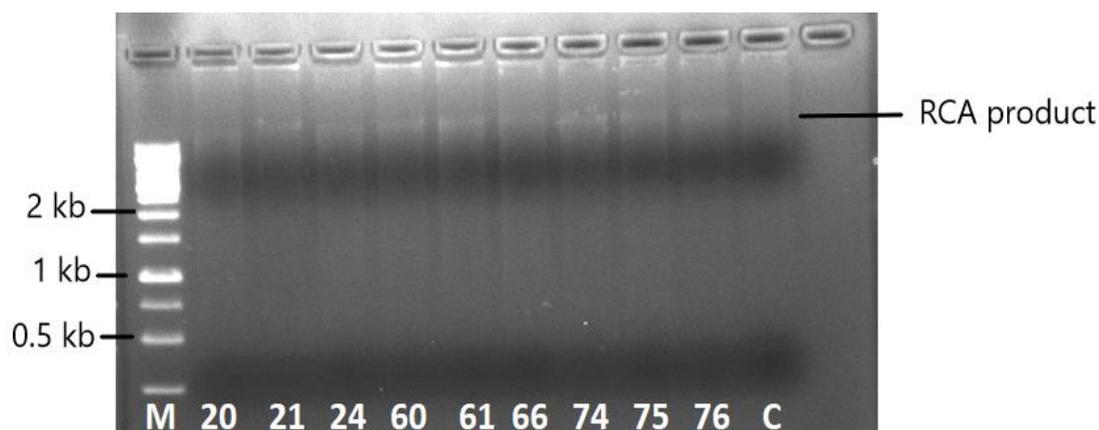


Figure 5: Agarose gel electrophoresis of RCA products. The lanes represent different leafhopper samples. Lane M is GeneRuler™ 1kb DNA Ladder (Thermo Scientific). Lane C is a negative control (MilliQ water) for RCA.

#### 4.3.1. Restriction enzyme analysis of RCA products

The RCA products shown in Figure 5 were digested with HindIII, EcoRI or SacI in separate reactions. Digestion with HindIII yielded bands of 0.7 kb, 1.4 kb, 1.7 kb and 1.9 kb for sample 21 (Figure 6), while for the other samples, no digestion products were obtained (data not shown). Digestion with EcoRI yielded fragments of 2.5 kb for sample 61 and 5.4 kb for sample 60 (Figure 6) while for the other samples, no digestion products were obtained (data not shown). Digestion with SacI yielded a fragment of 3 kb for sample 61 (Figure 6), while for the other samples, no digestion products were obtained (data not shown).

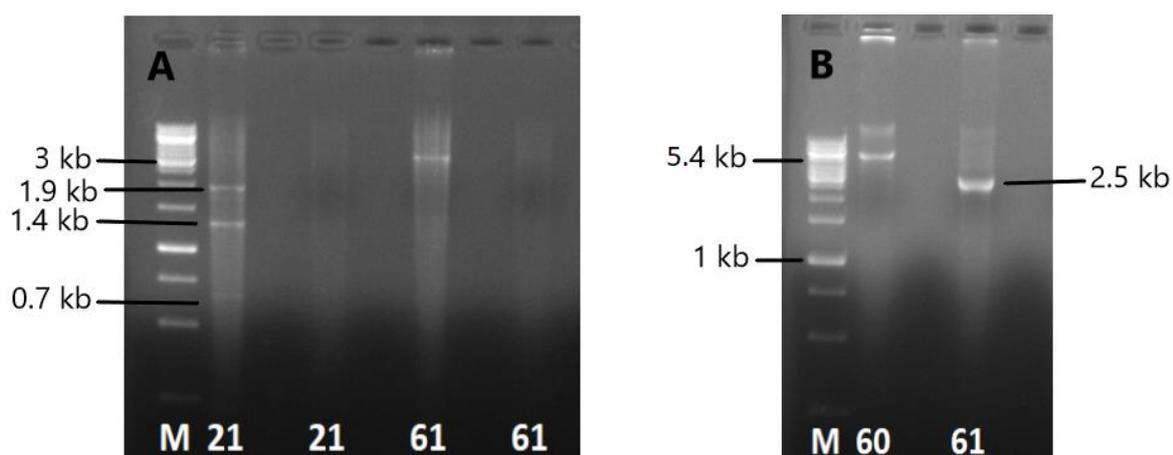


Figure 6: Agarose gel electrophoresis of RCA products digested with restriction enzymes. Lanes one and two in panel (A) are RCA product of sample 21 digested with HindIII and yielding fragments of 0.7 kb, 1.4 kb, 1.7 kb and 1.9 kb. Lanes three and four in panel (A) are RCA product of sample 61 digested with SacI and yielding a fragment of 3 kb. Lane one in

panel (B) is RCA product of sample 60 digested with EcoRI and yielding a fragment of 5.4 kb. Lane two in panel (B) is RCA product of sample 61 digested with EcoRI and yielding a fragment of 2.5 kb. Lane M is GeneRuler™ 1kb DNA Ladder (Thermo Scientific).

The fragments obtained after restriction digests of RCA were cloned and restriction digests of purified plasmids showed that some of the clones contained an insert of the expected size while some did not (Figure 7). Three clones of sample 21 (Lanes 1, 2 and 3) have insert with sizes of 700 bp. Three clones of sample 21 (Lanes 4, 5 and 6) have insert with sizes of 1.4 kb. Three clones of sample 60 (Lanes 10, 11 and 12) have insert with sizes of 5.4 kb. Three clones of sample 61 (Lanes 13, 14 and 15) have insert with sizes of 2.5 kb.

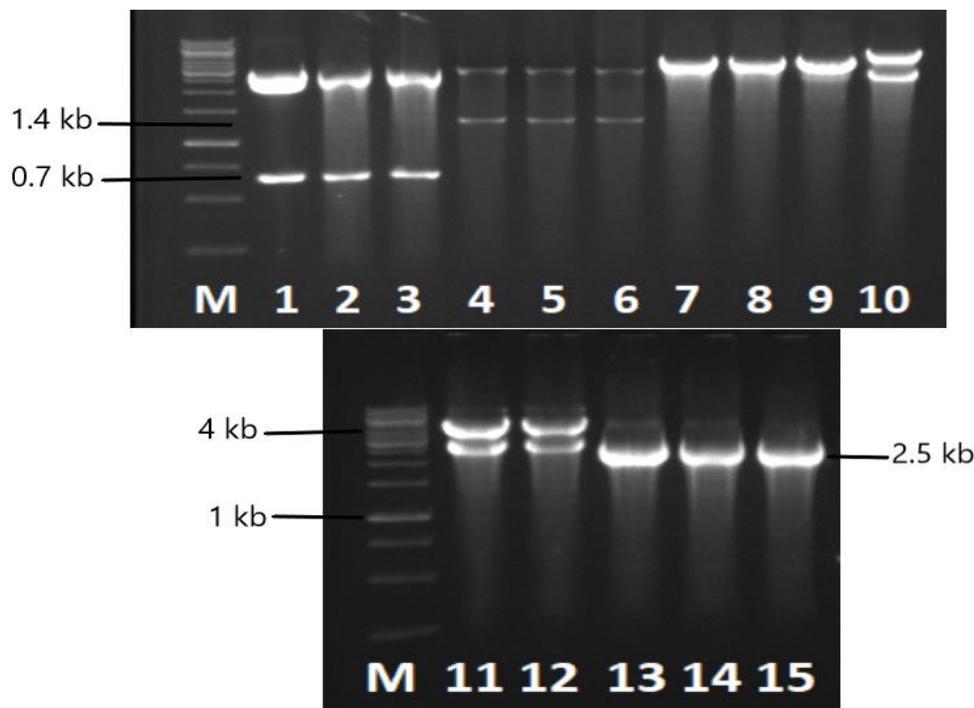


Figure 7: Agarose gel electrophoresis of plasmid DNA digested with restriction enzymes. Lanes 1 to 9 show results for clones of sample 21 digested with HindIII, lanes 10 to 12 results for clones of sample 60 digested with EcoRI and lanes 13 to 15 results for clones of sample 61 digested with EcoRI. Lane M is GeneRuler™ 1kb DNA Ladder (Thermo Scientific).

#### 4.4. Sequence analyses of cloned RCA products

The determined DNA sequences of the cloned RCA products were analyzed using Blastn and Blastx and compared with sequences in the GenBank and protein databases. Interestingly, the

complete insert of one clone (60\_1) from leafhopper sample 60 shared 83% nucleotide identity with WBDP plasmids (accession numbers JX668987.1 and JX668988.1) reported from China. This phytoplasma has been shown as a pathogen of wheat causing wheat blue dwarf disease (Zhang *et al.*, 1996; Chen *et al.*, 2014b).

Translation of the ORFs identified in the complete sequence (5438 bp) of the WBDP plasmid gave amino acid sequences of putative proteins with high identity to proteins of WBDP plasmid and *Candidatus* phytoplasma oryzae (CPO) (Table 3). The identified putative proteins were: replication-associated protein, effector protein, hypothetical protein, DUF2963 domain-containing protein and single-stranded DNA-binding protein (Figure 8). These proteins are also encoded by plasmids of other phytoplasmas.

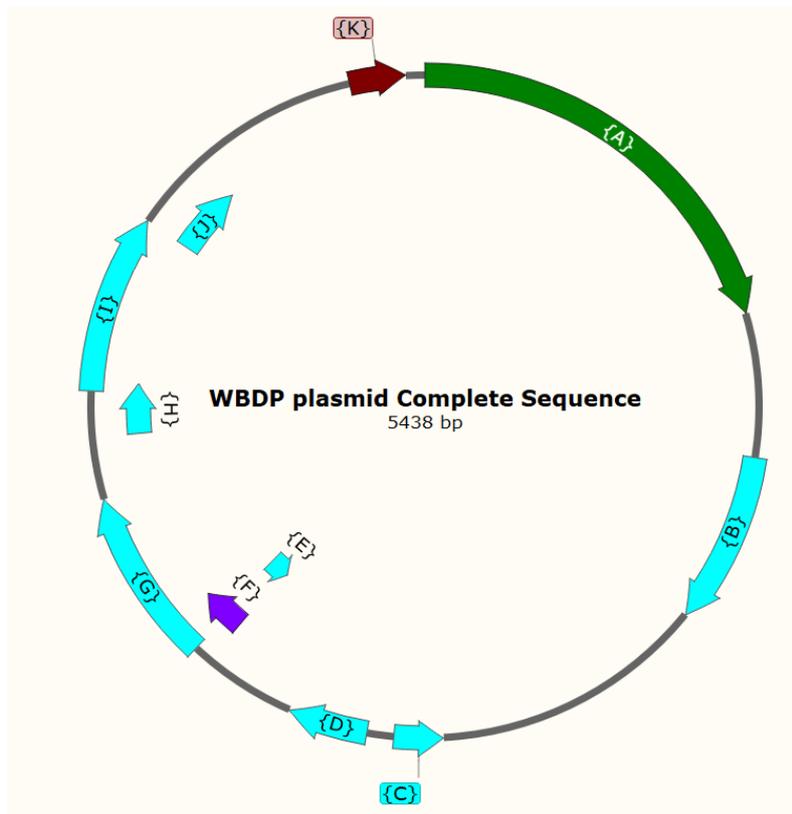
Table 3: The putative proteins encoded by the identified WBDP plasmid

ORFs	Gene-encoded	Query cover	Identity	Accession number
A	Replication associated protein	98%	76% to WBDP	WP_015083755
B	Hypothetical protein	78%	66% to CPO	WP_111961388
C	Hypothetical protein	76%	55% to WBDP	WP_015083760
D	Hypothetical protein	90%	78% to WBDP	WP_015083756
E	Hypothetical protein	70%	84% to WBDP	WP_015083761
F	DUF2963 domain-containing protein	62%	89% to WBDP	WP_024563532
G	DUF2963 domain-containing protein	96%	54% to WBDP	WP_015083757
H	Hypothetical protein	46%	100% to WBDP	WP_024563528
I	Hypothetical protein	99%	100% to WBDP	WP_024563528
J	Hypothetical protein	97%	100% to WBDP	WP_038251703
K	Single-stranded DNA-binding protein	97%	98% to WBDP	WP_024563530

WBDP = Wheat blue dwarf phytoplasma; CPO = *Candidatus* phytoplasma oryzae

Table 4: ORFs and putative proteins in the complete sequence of the WBDP plasmid

ORFs	Encoded protein	length	Position	Direction
A	Replication-associated protein	1113 bp	1 to 1113	Forward
B	Hypothetical protein	453 bp	1494 to 1946	Forward
C	Hypothetical protein	129 bp	2672 to 2800	Reverse
D	Hypothetical protein	210 bp	2871 to 3080	Forward
E	Hypothetical protein	81 bp	3309 to 3389	Reverse
F	DUF2963 domain-containing protein	135 bp	3325 to 3459	Forward
G	DUF2963 domain-containing protein	462 bp	3372 to 3833	Forward
H	Hypothetical protein	150 bp	3998 to 4147	Forward
I	Hypothetical protein	474 bp	4120 to 4593	Forward
J	Hypothetical protein	210 bp	4593 to 4802	Forward
K	Single-stranded DNA-binding protein	147 bp	5242 to 5388	Forward

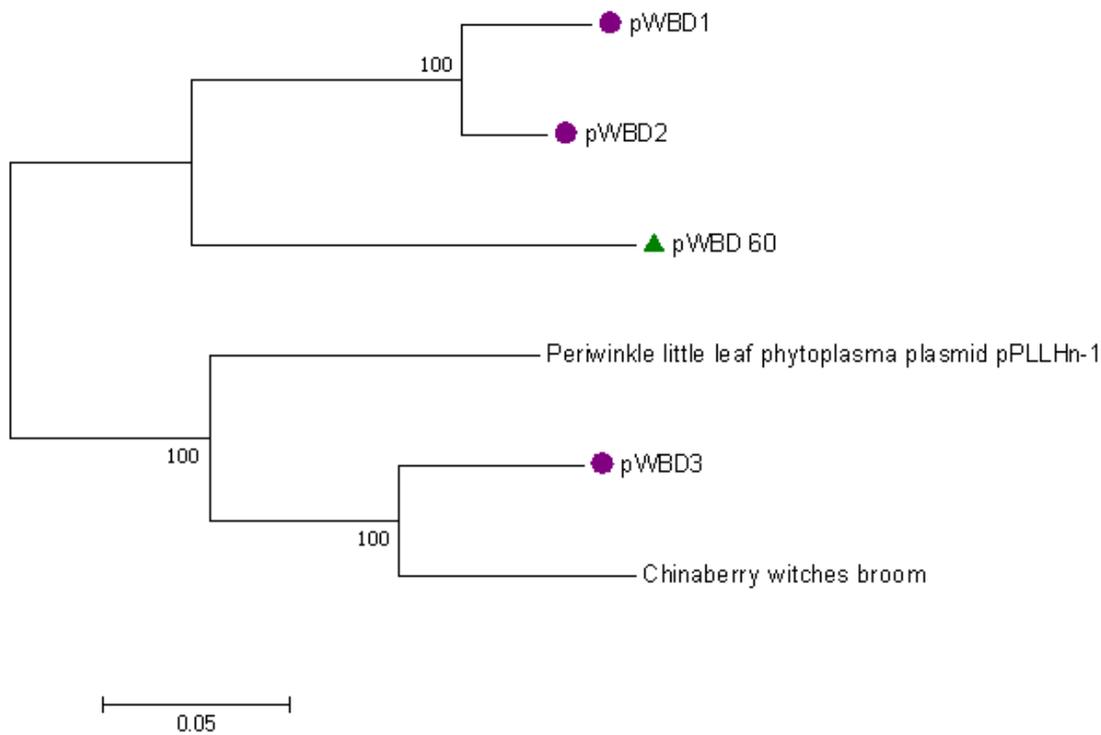


**Figure 8:** Sequence organization of the WBDP plasmid. The open reading frames (ORFs) are shown as forward and reverse arrows in the DNA line. The names and length of putative proteins are indicated in Table 4. Unlabelled DNA line represents non-coding DNA sequence. The complete size of the WBDP plasmid is 5438 bp.

In the phylogenetic analysis, WBDP plasmid sequences from GenBank were used as indicated in Table 5. A region of 1125 bp covering part of the replication-associated protein gene and non-coding DNA was used for phylogenetic analysis. The results revealed that the WBDP plasmid identified in this study was closely related to WBDP plasmids (accession numbers JX668987.1 and JX668988.1) reported from China (Figure 9).

Table 5: Description of phytoplasmal plasmids used for phylogenetic analysis

Plasmid name	Country	Accession number
Wheat blue dwarf phytoplasma 1 (pWBD 1)	China	JX668987
Wheat blue dwarf phytoplasma 2 (pWBD 2)	China	JX668988
Wheat blue dwarf phytoplasma 3 (pWBD 3)	China	JX668989
Periwinkle little leaf phytoplasma pLLHn-1	China	JN835187
Chinaberry witches'-broom phytoplasma	China	JF827298
Wheat blue dwarf phytoplasma 60 (pWBD 60)	Sweden	Unpublished



**Figure 9:** Phylogenetic analysis of 6 phytoplasmal plasmids based on the alignment of 1125 bp covering part of the replication-associated protein gene and non-coding DNA. The figure shows a neighbour-joining tree inferred from Kimura two-parameter estimates of nucleotide substitutions per site. The WBDP plasmid identified in this study is marked with a triangle and WBDP plasmids from China with circles. Numbers above branches represent bootstrap values out of 1000 replicates. Only bootstrap values over 60 are shown. Branch length is drawn to scale with the bar indicating 0.05 nt substitutions per site.

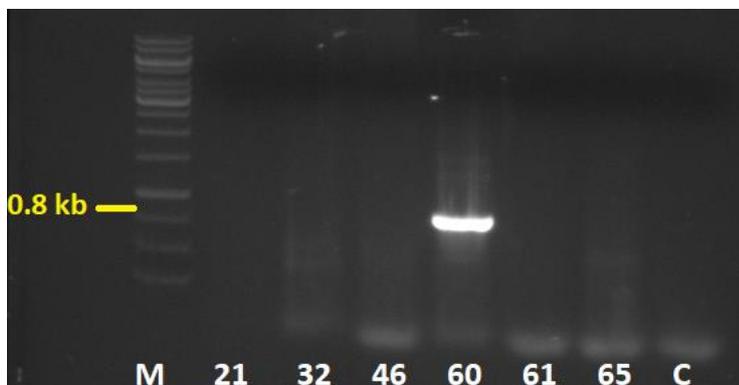
In addition, sequences for other RCA products were analysed, for example, the sequences (700 bp) of three clones from leafhopper sample 21 shared 89% nucleotide identity with transmissible spongiform encephalopathy (TSE) associated circular DNA isolate Sphinx 2.36 (accession number HQ444405). Also, they shared 85% nucleotide identity with *Acinetobacter baumannii* strain DS002 plasmid pTS236 (accession number JN872565). These sequence results are consistent with those for another sample of leafhoppers obtained previously in a preliminary experiment.

The sequence from one clone (1.4 kb) for sample 21 shared 92% nucleotide identity with *Yersinia enterocolitica* cryptic plasmid p29807 (accession number AJ132618). This plasmid was reported to be used for the construction of cloning vectors (Strauch *et al.*, 2000). The

sequence from one clone (2.5 kb) for sample 61 shared 72% nucleotide identity with *Pseudomonas koreensis* strain P19E3 plasmid p4 (accession number CP027481).

#### 4.5. PCR developed to detect plasmid of wheat blue dwarf phytoplasma

Fourteen leafhopper samples positive for WDV were tested also for the presence of WBDP by PCR using specific primers. Following agarose gel electrophoresis of PCR products, the expected fragment with the size of 895 bp was obtained only for leafhopper sample 60 (Figure 10). This confirmed that the sample consisting of several leafhopper individuals carried virus and phytoplasma pathogens which both have been reported to cause dwarf disease in wheat. Also, this result is consistent with the result obtained from RCA and sequence data that together confirm the presence of WBDP plasmid in the leafhopper *P. alienus*.



**Figure 10:** Agarose gel electrophoresis of PCR products. The lanes represent different leafhopper samples. A molecular band with the size of 895 bp for leafhopper sample 60 is indicating the presence of wheat blue dwarf phytoplasma plasmid. Lane **M** is GeneRuler™ 1kb DNA Ladder (Thermo Scientific). Lane **C** is a negative control (MilliQ water) for PCR.

## 5. Discussion

### 5.1. Identification of WDV in leafhoppers

In previous studies, WDV has been identified in host plants and in the insect vector *P. alienus* from different regions around Sweden (Kvarnheden *et al.*, 2002; Ramsell *et al.*, 2008). Following the outbreak of WDD in Sweden in 2017 with the occurrence of leafhoppers in the affected regions, we also characterized WDV in the insect vector *P. alienus* collected in autumn 2017 from different parts of Sweden. Through PCR using WDV-specific primers (Kvarnheden *et al.*, 2002), WDV was detected in different samples of leafhoppers. These primers have been shown to be able to detect a wide range of WDV isolates (Ramsell *et al.*, 2008). Using blast nucleotide analysis, the obtained sequences were found to share 99% nucleotide identity with

previously sequenced WDV isolates from Sweden. In phylogenetic analyses, the WDV isolates identified in this study showed a close relationship to WDV isolates previously reported from Sweden. These results are consistent with the results of the phylogenetic analysis demonstrated by Ramsell *et al.* (2008), where, all of the WDV isolates from Sweden grouped into one clade. The data from the present study indicate that the disease outbreak in 2017 was caused by similar isolates of WDV as previously reported from Sweden. Moreover, for understanding the genetic diversity of complete genomes of WDV, further sequencing of isolates obtained in this study is suggested with the use of primers for amplifying complete sequences.

## **5.2. Identification of viruses using RCA**

The study of viruses with genomes of circular DNA has been simplified due to the use of the method of rolling circle amplification (RCA), which amplifies the complete virus genome. Previously, the method has been used to amplify the complete genome of WDV (Schubert *et al.*, 2007; Ramsell *et al.*, 2009) and was successful also in this study to amplify circular DNA from *P. alienus* DNA extracts. However, in the present study WDV was not identified from amplified RCA products. Probably, the DNA concentration of WDV was too low to be identified compared to the DNA of other viruses or plasmids in the RCA products. RCA has shown to have high proofreading by providing more accurate genome sequences than common PCR (Wang *et al.*, 2014a). Moreover, this method has become common for identification of different potential pathogens with circular DNA in the host plant as well as the vector (Inoue-Nagata *et al.*, 2004; Demidov, 2016). Using RCA, it is possible to amplify the circular DNA of viruses, bacteriophages and plasmids.

In the present study, RCA was a successful method for obtaining circular DNA of a virus that shares 89% nucleotide identity with transmissible spongiform encephalopathy (TSE)-associated circular DNA isolate Sphinx 2.36 and 85% nucleotide identity with *Acinetobacter baumannii* strain DS002 plasmid pTS236. Sphinx was found to co-purify with prions and the titre of Sphinx was higher in prion-infected cells (Manuelidis, 2011). However, prions are reported to cause TSE in mammals including humans (Manuelidis, 2011; Longkumer *et al.*, 2013). According to studies by Manuelidis (2011) and Longkumer *et al.* (2013), an *Acinetobacter* phage genome is similar to Sphinx 2.36. Different studies also have shown that *Acinetobacter* spp are present in water (Aguirre de Carcer *et al.*, 2015), soil samples (Longkumer *et al.*, 2013) and in the gut of aphids (Engel & Moran, 2013). These results are

indicating that the identified type of bacteriophage is common in the environment and may be acquired by leafhopper through feeding on plants.

### 5.3. Identification of plasmids using RCA

The unique advantage of using the RCA method is that it can amplify circular DNA molecules of different microorganisms. For example, among the circular DNA molecules amplified in this study was a WBDP plasmid with the size of 5.4 kb. The sequence of the WBDP plasmid matched throughout its length to sequences of WBDP plasmids from China (accession number JX668987, JX668988 and JX668989) and showed an overall nucleotide identity of 83% to these WBDP plasmids. In China three plasmids of WBDP have been discovered with the length of 3449 bp, 3601 bp and 3844 bp (Chen *et al.*, 2014b). A phylogenetic analysis based on a region of 1125 bp covering the replication-associated protein gene and non-coding DNA confirmed that the WBDP plasmid identified in this study was closely related to the WBDP plasmids from China.

WBDP has also become of interest in this study because it is a bacterium that can cause blue dwarf disease in wheat. It has been reported to cause WBDD in China with severe yield losses in wheat at different occasions (Wu *et al.*, 2010; Chen *et al.*, 2014a; b). In Sweden, there are no reports showing detection of WBDP in the insect vector *P. alienus* or in wheat crops (Kvarnheden A; personal communication). There are also no reports of WBDD. In Sweden, the yellow-type disease in oat, rye and barley was also shown by Lindsten *et al.* (1970) through transmission tests with the leafhopper *Macrostelus laevis* and it was suspected that a bacterium termed as mycoplasma-like organism (MLO) was the agent of the disease. The symptoms of the yellow-type disease shown in these transmission experiments were similar to symptoms caused by WBDP on wheat reported from China. At that time, there was no confirmation if the pathogen was mycoplasma or virus, and therefore they continued to consider yellow-type disease to be caused by virus (Lindsten *et al.*, 1970). The sample of *P. alienus* that was shown to contain WBDP was collected from Ringarum in the county of Östergötland. Interestingly, a yellow-type disease had been found to be transmitted by *M. laevis* leafhoppers collected in the same part of Östergötland county (Lindsten *et al.*, 1970), and the present results indicate that WBDP may still be present there.

Furthermore, organisms of the genus *Phytoplasma* have small genomes with a very low percentage of G and C nucleotides (Bertaccini & Duduk, 2010; Oshima *et al.*, 2013). This low

GC content is consistent with the sequence of the WBDP plasmid (5438 bp) obtained in this study, that had a GC content of 23%.

The phytoplasmal pathogens are transmitted by leafhoppers (Cicadellidae), psyllids (Psyllidae) and planthoppers (Fulgoroidea)(Weintraub & Beanland, 2006; Wu *et al.*, 2010). In China, WBDP is known to be transmitted by the leafhopper *Psammotettix striatus* (Chen *et al.*, 2014a; b). Following observation under electron microscopy, phytoplasma were found in the phloem tissues of WBDP-infected wheat and in the saliva tissue of *P. striatus* (Wu *et al.*, 2010). Like other phytoplasma, WBDP is an obligate pathogen that depends on plant host and insect vector for survival and multiplication (Qinfeng *et al.*, 1996; Weintraub & Beanland, 2006). WBDP has a wide host range including plants of the families Poaceae, Brassicaceae and Solanaceae (Gu *et al.*, 2007; Wu *et al.*, 2010). Common symptoms, which occur in plants due to WBDP infections, are yellowing of leaf tips, virescence, reddening, phyllody, stunting, witches' broom, dark blue-green colour in stem and reduced seed set due to sterile flowers (Wu *et al.*, 2010; Chen *et al.*, 2014a; Wang *et al.*, 2018). Previously, WBDD was known as wheat red dwarf disease (WRDD) due to the reddening symptom it induces in the plant, but later it was changed to WBDD to differentiate it from wheat yellow dwarf disease (WYDD) caused by barley yellow dwarf virus (BYDV) and cereal yellow dwarf virus (CYDV) (Zhang *et al.*, 1983, 1996; Wu *et al.*, 2010).

For future studies, testing is suggested of host plants around Östergötland county which will give an understanding of how common these phytoplasma are in that area. In this study, the detection of WBDP in the leafhopper *P. alienus*, which is also a vector of WDV, is reported.

## **6. Conclusion**

Generally, this project demonstrated that RCA is able to assist in detecting a range of viruses and plasmids. To my understanding, this will be the first report to show the detection of WBDP in the leafhopper *P. alienus* in Sweden. Interestingly, these phytoplasma were identified from a sample containing leafhoppers collected from a field in Östergötland county, previously, *M. laevis* leafhoppers collected from that region were also demonstrated to carry phytoplasma that cause yellow-type disease. This raised an idea that probably the WBDP may still be there and further studies on leafhoppers and plant samples from Östergötland county will provide a clear understanding of WBDP in that region. In addition, the results of this study show that there are no new genotypes of WDV and the outbreak of WDD in 2017 was caused by isolates of WDV closely related to those previously reported from Sweden. Also, detection of sphinx in *P.*

*alienus* is indicating that this bacteriophage is common in the environment and may be acquired by leafhoppers through feeding on different plants.

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