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# Diversity of barley yellow dwarf-associated viruses in winter cereals of southern Sweden

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### Abstract

Barley yellow dwarf virus (BYDV) is a widespread complex of virus species of cereal crops in the world, known to infect both wild and cultivated plants of the family Poaceae, having the effect of yield reductions and subsequently large economic losses to farmers. Barley yellow dwarf disease (BYD) is a result of infection from BYDV species (genus: *Luteovirus*) and cereal yellow dwarf virus (CYDV; genus: *Polerovirus*). The symptoms of infection include the yellowing of leaf tips, rolling of leaf margins, stunting of plants, reduction in seed set and fertility. Taxonomically the viruses causing disease are the luteovirus species BYDV-MAV, BYDV-PAV and BYDV-PAS as well as the polerovirus CYDV-RPV. These are positive sense single-stranded RNA (+ssRNA) viruses vectored by different aphid species, including *Sitobion avenae*, *Rhopalosiphum padi*, *Schizaphis graminum* and *Metopolophium dirhodum* in a circulative nonpropagative manner. In Sweden, seasonal field surveys are conducted along with monitoring of aphid species to determine seasonal outbreak scenarios. In spring 2015 and 2017, BYD was confirmed from preliminary field surveys, identifying BYDV-PAV, BYDV-MAV and CYDV-RPV. This study was carried out to determine the viral diversity of isolates collected from plant material in southern Sweden, as well as to investigate the possible presence of phenotypic mixing or transcapsidation during infection in winter barley (*Hordeum vulgare*), winter wheat (*Triticum aestivum*) and winter rye (*Secale cereale*). DAS-ELISA and PCR amplification of the BYDV coat protein (CP) genes was used to determine viral diversity and the presence of phenotypic mixing or transcapsidation. Through sequence analyses of cloned PCR products, BYDV-PAV and BYDV-PAS were identified as viruses causing disease during outbreaks in spring 2015 and 2017. The BYDV-PAV isolates were closely related to previously recorded isolates of BYDV-PAV in Sweden, Latvia and Germany. In a phylogenetic analysis, Swedish and global BYDV-PAS isolates formed a well-supported group indicating that BYDV-PAS is a widespread virus. In addition, an isolate of Barley virus G, a virus first identified in barley of South Korea was identified in Swedish barley from the disease outbreak of 2015 and represents the first record of the virus in Sweden. DAS-ELISA results identified BYDV-PAV, BYDV-MAV and CYDV-RPV as viruses causing BYD disease in the outbreaks of 2015 and 2017, differing from that of CP gene sequence analysis which did not identify BYDV-MAV or CYDV-RPV. The results suggest that antibodies for BYDV-PAV and BYDV-MAV detect the CP of BYDV-PAS, because samples were most often positive for both BYDV-PAV and BYDV-MAV in DAS-ELISA, but BYDV-PAV and BYDV-PAS were detected by sequence analyses of the CP gene. Antibodies for CYDV-RPV seem to detect Barley virus G because of unspecific detection by DAS-ELISA antibodies for BYDV-PAV and BYDV-MAV, unable to adequately differentiate the CPs of these species. Future work should focus on ELISA antibody development for discrete identification of BYD virus species.

*Keywords:* BYDV, barley, CYDV, diversity, phenotypic mixing, transcapsidation, wheat

## Popular science summary

Globally barley yellow dwarf (BYD) disease is the most widespread viral disease of cereals, known to affect many economically important crops such as barley, wheat, oat and rye. In agricultural fields, BYD disease is caused by the barley or cereal yellow dwarf viruses (B/CYDVs), including the three common species of *Barley yellow dwarf virus-PAV* (BYDV-PAV), *Barley yellow dwarf virus-MAV* (BYDV-MAV) and *Cereal yellow dwarf virus-RPV* (CYDV-RPV). These are a group of closely related but distinct viruses of the genera *Luteovirus* (BYDV-PAV and BYDV-MAV) and *Polerovirus* (CYDV-RPV). BYD disease is identified by plane aerial surveys, visible in fields as mixed yellowing patches with dwarfed plants. Up close, plants have yellowed leaves, rolled leaf tips and uneven signs of dwarfing. B/CYDVs are vector transmitted by aphids, flying insects feeding by sucking sugar containing sap from plants and taking up the virus, before moving onto healthy plants and infecting these. Disease patches are randomly spread in fields, a consequence of short aphid flight distances. Within disease patches, infections may be of single or mixed virus species. An example of a common infection combination is BYDV-PAV and BYDV-MAV yet triple infections are rare. Aphid vectors include; the English grain aphid (*Sitobion avenae*), bird cherry-oat aphid (*Rhopalosiphum padi*), wheat aphid (*Schizaphis graminum*) and rose-grain aphid (*Metopolophium dirhodum*). These are common aphid species across the world and are in part responsible for the large geographic distribution of these viruses. B/CYDVs have specific virus-vector relationships, where transmission of each virus species is either unique to an aphid species or aphids have differing efficiencies for each virus. This specificity is due to the coat protein (CP), a protein building up the coat, which encloses each virus ensuring protection of nucleic acid. It is hypothesised that amongst the B/CYDVs there is exchange of the whole coat or interchange of smaller CP building blocks between viruses. Both of which may increase the number of vectors per virus and facilitate transmission of multiple viruses. In spring 2015 and 2017 outbreaks of BYD disease occurred in southern Sweden and Denmark. From these outbreaks, the diversity of virus species and potential of CP exchange or interchange was investigated in winter barley (*Hordeum vulgare*), winter wheat (*Triticum aestivum*) and winter rye (*Secale cereale*). Broad B/CYDVs identification by diagnostic testing, capturing virus particles CP from leaves, coupled with molecular techniques for sequence analysis of the CP gene allowed for virus species identification. Diagnostic testing identified two predominant virus species in 2015 and 2017, BYDV-PAV and BYDV-MAV and to a lesser extent CYDV-RPV. Analysing the CP gene identified BYDV-PAV and barley yellow dwarf virus-PAS (BYDV-PAS) and no identification of BYDV-MAV or CYDV-RPV. Using CP gene sequence comparisons, the evolutionary relationship between B/CYDV isolates from 2015 and 2017, and previously identified Swedish and global isolates was determined. Swedish BYDV isolates divided into three groups: firstly, a well-supported BYDV-PAS group, of 2015 and 2017 Swedish isolates along with isolates from USA and Germany. Secondly, BYDV-PAV isolates from Sweden and Latvia and thirdly a group of solely Swedish and Latvian BYDV-MAV isolates. Barley virus G was identified in winter barley from the 2017 disease outbreak in southern Sweden, a virus previously only recorded in South Korea and recently in the USA. The presence of CP exchange or subunit interchange in isolates from 2015 and 2017 outbreaks was not confirmed.

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# 1 Introduction

## 1.1 Symptoms of barley yellow dwarf disease

Barley yellow dwarf (BYD) disease has a widespread occurrence globally (Domier, 2009; Lapierre, 2004; Lister and Ranieri, 1995). It is known to affect many cultivated and wild grass species of the family Poaceae (D'Arcy, 1995; Shah et al., 2012). BYD disease is a result of infection from a complex of viruses within the family *Luteoviridae*, either the barley yellow dwarf viruses (BYDVs) or cereal yellow dwarf viruses (CYDVs) and they are transmitted in a distinct manner by different aphid vector species (Gray and Gildow, 2003; Miller and Rasochová, 1997). BYD poses a great risk in cultivated food crops, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and oat (*Avena sativa*) where its effect threatens production (Adachi et al., 2015; Shah et al., 2012).

Symptoms of BYD disease are heavily influenced by the crop and cultivar type, environmental conditions and the developmental and physiological status of plants upon infection (D'Arcy, 1995; Kaddachi et al., 2014). One example of the environmental influence on symptom development, is that of lower temperatures, when leaf reddening becomes a prominent feature in affected wheat plants (Shah et al., 2012). Disease symptoms are typified by the presence of yellowed leaf discoloration and reduced plant growth, resulting in stunting or dwarf phenotypes (Rochow, 1970a). Leaf discoloration is variable between crop types, most often present as yellowing of leaf tips and margins (Lapierre, 2004). Yellowing of leaves in barley is known to be unevenly distributed along the surface of the leaf blade, making it identifiable in the field to the studied eye (Shah et al., 2012). Stunting and dwarfing of infected plants is one of the most economically important disease symptoms, most notably in barley and oat (Domier, 2009). In barley, infection is associated with increased rigidity in leaves, reddening of leaf margins, delayed tiller formation, reductions in grain weight and sterility of florets (D'Arcy, 1995; Kaddachi et al., 2014; Miller and Rasochová, 1997). Other symptoms of BYD disease are decreased root development, serration of leaf margins and distortions to leaf folding (Choudhury et al., 2017; D'Arcy, 1995). BYD symptoms are difficult to identify, especially as leaf discoloration may be due to plant nutrient status or other pathogenic action (D'Arcy, 1995; Miller et al., 2002).

## 1.2 Economic aspects of barley yellow dwarf disease in cereal crops

Currently there is no global estimate on the economic value of losses as a result of BYDV infections, however there are regional reports indicating crop losses which may vary from 11 – 86% (Miller and Rasochová, 1997). In oats, BYDV is known to cause extremely reduced seed set with subsequent severe yield losses (Miller et al., 2002). In 1969, BYDV infection in Canadian oats resulted in reductions in the number of seeds as well in the weight of harvested grains, where moderate infections gave significantly reduced yields per plot compared to healthy uninfected plants (Martens and McDonald, 1970). An assessment of yield losses due to BYDV infection in wheat and oat during the years 1992-1994 in Australia, found that losses occurred

in a range of 1300 – 2700 kg/ha, described by a linear relationship between incidence of BYDV infection and decreased yield (McKirby et al., 2002).

Surveys during 1995-1997 on BYDV-PAV infection of red winter wheat in Indiana and Illinois showed that with increased BYDV incidence in fields there was a reduction in yield of 27 – 45 kg/ha per percent BYDV infection detected (Perry et al., 2000). Losses also had a seasonal component, in which seeds sown in early spring had higher yield reductions in comparison to autumn-sown crops. An Australian study on BYDV-PAV infection in wheat during 1991 – 1993, showed that later sown plants had higher yields, with differences of 2793 kg/ha for sowing in early May compared to 1543 kg/ha for late June (McKirby and Jones, 1997). A 7 year assessment of BYDV infection effects on yield aspects of winter wheat during 2005 – 2013, using modelling techniques, found that BYDV infection was responsible for ~30% reductions in yield (Gauce and Bockus, 2015). Recorded disease incidence and yield reductions were linearly correlated, with highest yield loss of 85% in 2005 and the lowest at 25% in 2011. A 10-year study in northern France during 1989 – 1999 studied BYDV infection in barley and analysed aphid activity and symptom development to develop a regression model to estimate the cost of BYDV infection and aid decision-making (Fabre et al., 2003). The model was able to determine a threshold of 500 kg/ha estimated loss as a tipping point for deciding on insecticide application during BYD disease outbreaks.

In Ireland, BYDV infection of spring barley sown in March -April was investigated during 1990 – 1993 and 1996-2001 for yield effects (Kennedy and Connery, 2005). Aphicides, of either pyrethroid or organophosphorous-type were applied to plants throughout development to determine the possible effect they may have in reducing infection, symptom development and yield losses (Kennedy and Connery, 2005). The results from this assessment showed that there was higher occurrence of aphids during April for the study period 1991-1993 and 1996-2001, with *Sitobion avenae* as the most abundant aphid followed by *Metopolophium dirhodum*. BYDV-MAV was identified as the most prevalent cause of disease, with affected plants displaying symptomatically yellow leaves. The most effective aphicide treatment for reducing symptom formation at all development phases was with pyrethroid based agents (Kennedy and Connery, 2005). BYDV-infected plants, to which pyrethroid agents had been applied at varying doses, had a higher number of tillers compared to infected plants treated with organophosphorous-type agents. When estimating the yield losses during the study period, there was a reduction of 0.27 t/ha for barley planted in March (Kennedy and Connery, 2005).

When combining the outputs from these studies it is clear that yield losses due to BYD may be variable, with a strong influence by cultivar, seasonal sowing time and yearly aspects (Gauce and Bockus, 2015; McKirby and Jones, 1997; Perry et al., 2000).

### 1.3 Taxonomy and genome organization of barley yellow dwarf and cereal yellow dwarf virus

The taxonomy of BYDV has undergone several changes, early classification systems were based primarily on serological studies, using antibodies for detection (Mayo and D’Arcy, 1999). More recent classification schemes have utilised both molecular and sequencing techniques to categorise viruses causing BYD based on coat protein (CP) sequence identity, genomic structure organization with open reading frames (ORFs) and protein translation mechanism (Domier, 2010, 2011; Mayo and D’Arcy, 1999). Presently, BYD-associated viruses are classified as belonging to the family *Luteoviridae*, which is comprised of the three genera *Luteovirus*, *Polerovirus* and *Enamovirus* (Domier, 2011). BYDVs included within the genus *Luteovirus* and associated with BYD disease are: *Barley yellow dwarf virus-MAV* (BYDV-MAV), *Barley yellow dwarf virus-PAS* (BYDV-PAS), *Barley yellow dwarf virus-PAV* (BYDV-PAV) (Lefkowitz et al., 2017). The closely related viruses *Cereal yellow dwarf virus-RPV* (CYDV-RPV) and *Cereal yellow dwarf virus-RPS* (CYDV-RPS) belong to the genus *Polerovirus*, differing from viruses in the genus *Luteovirus* in the genome organization at the 5’ end (Domier, 2011; Lefkowitz et al., 2017; Mayo and Miller, 1999) (Figure 1). The

genomes of luteovirid viruses are in the range of 5600 – 5900 nucleotides (Domier, 2011; Miller et al., 2002), with luteovirus genomes containing 6 ORFs (Domier, 2011). The ORFs of viruses of the genera *Luteovirus*, *Polerovirus* and *Enamovirus* are similarly arranged and encode viral proteins via the synthesis of subgenomic RNAs (sgRNA) for each ORF (Domier, 2011; Miller et al., 1997). Luteovirus genomes contain ORF1 – ORF6, whilst polerovirus and enamovirus genomes include ORF0 – ORF5 and the presence of a genome linker viral protein (VPg) at the 5' end (Domier, 2011; Miller et al., 2002). The regular genome organization between *Luteoviridae* virus species is also shown in the functions of each ORF, such that ORF0 which is present in polerovirus and enamovirus genomes encodes a protein for RNA silencing suppression (Domier, 2011; Liu et al., 2012). ORF1 of viruses in the genera *Polerovirus* and *Enamovirus* encodes the VPg, whilst for the luteoviruses ORF1 encodes a protein with a helicase motif (Domier, 2011; Miller et al., 2002). Ribosomal frameshift of ORF1 and ORF2 leads to the translation of the RNA-dependent RNA polymerase (RdRP), ORF3 encodes the major coat protein (CP), ORF4 encodes the movement protein (MP) and translational readthrough of ORF3 and ORF5 leads to the translation of a readthrough domain (RTD) (Ali et al., 2014; Domier, 2011; Miller and Rasochová, 1997; Miller et al., 2002). ORF6 has recently been demonstrated for *Barley yellow dwarf virus-GAV* (BYDV-GAV), an unassigned member within the family *Luteoviridae*, to code for an RNA silencing suppressor (Liu et al., 2012). sgRNA's are used in the translational mechanisms for ORFs 3 – 6, whilst ORFs 1 - 2 are translated directly from luteovirid genomes (Domier, 2011). For viruses of the genus *Luteovirus*, sgRNA's use a cap-independent translation mechanism, due to the absence of a 5' cap and 3' poly-A tail (Miller et al., 2015). The cap-independent translation element (CITE) of BYDV is known as the BYDV-like translation element (BTE) and it is located in the upstream untranslated region at the 3' end of the genome (Banerjee and Goss, 2014; Kneller et al., 2006; Miller et al., 2015; Simon and Miller, 2013).

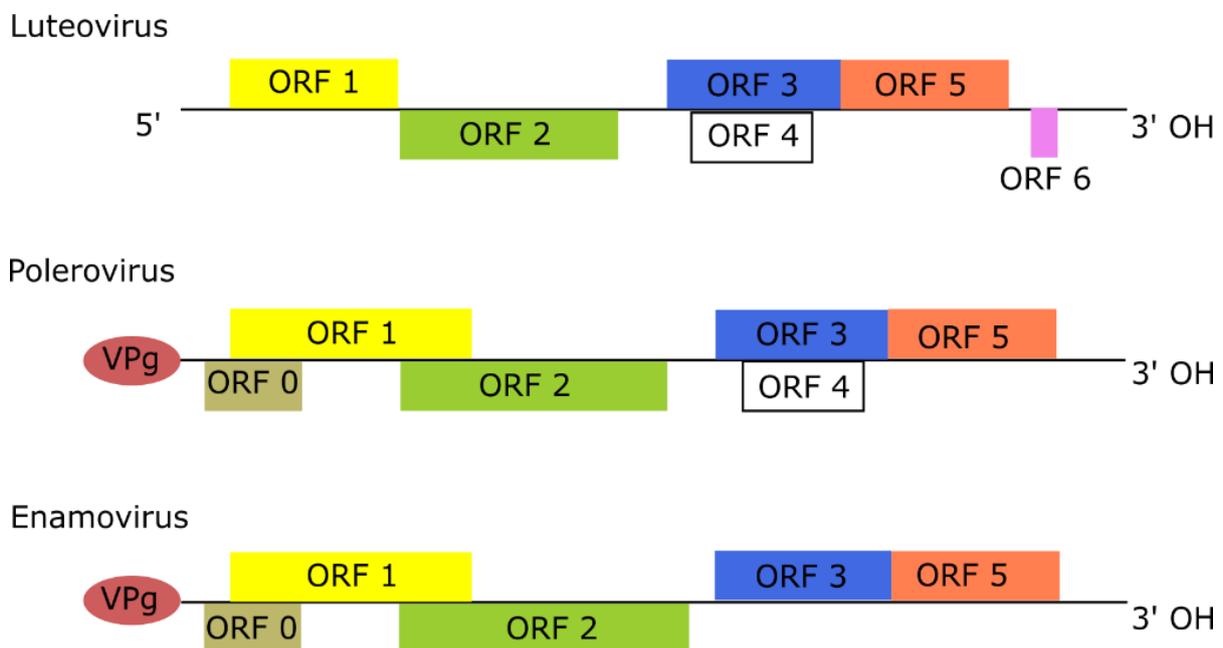


Figure 1. Viruses of the family *Luteoviridae* may be divided based on their genomic organization into three genera: *Luteovirus*, *Polerovirus* and *Enamovirus*. ORF0: RNA silencing suppressor, ORF1: VPg, ORF1 and ORF2: RdRP, ORF3: CP, ORF4: MP, ORF3 and ORF5: Minor coat protein, ORF5: RTD, ORF6: RNA silencing suppressor.

## 1.4 Vector specificity of barley yellow dwarf virus

BYD is an extensively studied system for vector specificity. This has demonstrated that key aphid species such as *S. avenae*, *Rhopalosiphum padi*, *Schizaphis graminum* and *M. dirhodum* interact with specific BYD-associated viruses, which are maintained internally within the aphid and transmitted to host plants (Brault et al., 2010; Gildow, 1999; Gray and Gildow, 2003). BYDVs are circulative nonpropagative viruses, thus they are characterised as being internally transported within vectors by traversing internal barriers, during which viral replication does not occur (Gildow, 1999). Viral replication occurs within the plant host, once the virus has been transmitted from the vector and thereafter the virus is transported within the phloem of infected plants (Ng and Perry, 2004). The internal transport of BYDVs within the vector involves passing through the food canal, foregut, midgut, hindgut, haemocoel, accessory salivary glands and finally to the salivary duct, where they exit into the host plant for infection (Gildow, 1999; Gray and Gildow, 2003; Power and Gray, 1995). The period, during which a virus is captured by the aphid from one host and transported internally and within the vector, before transmitted to another host is referred to as the latency period (Brault et al., 2010; Gray and Gildow, 2003). The latent period is characterised as a time when the virus is not transmitted, despite being contained within the vector and this period may persist for 1 – 4 days (Gray and Gildow, 2003). BYDVs have a highly specific interaction with aphid vectors, such that viruses may be transmitted by either one or two aphid species (Brault et al., 2007). The close knit interaction is maintained by the internal movement of the virus within the vector, with each structural component of the aphid acting as a selective barrier to viral movement (Brault et al., 2010, 2007; Ng and Perry, 2004). Thus, the transmission efficiency of aphids for each BYDV and CYDV is determined by the ability to take up virus, internally transport virus inside the vector and successful secretion to the next plant for infection (Gildow, 1999). Thus far, studies on the vector-specific relationships for BYDVs have shown that *R. padi* is able to transmit BYDV-PAV, BYDV-MAV with low efficiency, CYDV-RPV; *S. avenae* is a vector of BYDV-MAV and BYDV-PAV; *S. graminum* is a vector for CYDV-RPV and BYDV-PAV; *M. dirhodum* is a vector for BYDV-MAV and BYDV-PAV (Brault et al., 2007). To facilitate this specificity with vectors, it has been hypothesised that the virus capsid interacts with receptors in the aphid stylet and subsequently with receptors in the internal structures of the aphid (Brault et al., 2010). The wide host range of *R. padi* and *S. avenae* and their global distribution allows for the transmission of BYDVs and CYDVs to a wide array of wild and cultivated grass species (Ng and Perry, 2004).

## 1.5 Transcapsidation and phenotypic mixing of barley yellow dwarf viruses

BYDV infection has long been known to occur naturally in mixed infections of individual plants, thus there have been studies investigating the transmission system in which this is possible to occur (Rochow, 1970). This can occur via two processes, a virus is encapsidated with the CP of another virus, referred to as transcapsidation or a virus is encapsidated by CP from both viruses, known as phenotypic mixing. Both processes are collectively termed heterologous encapsidation (Creamer and Falk, 1990; Hu et al., 1988; Wen and Lister, 1991). Early studies sought to elucidate the vector-specific nature of such a relationship, using the transmission ability of *R. padi* and *S. avenae* towards BYDV-MAV and CYDV-RPV (Rochow, 1970b). These experiments used antisera against either BYDV-MAV or CYDV-RPV to block the ability of vectors to transmit the virus, which is regularly transmitted during mixed virus infections. Transmission tests with *A. byzantina* (Coast Black oats type) and two aphid species, *S. avenae* and *R. padi* showed that *S. avenae* was not able to transmit CYDV-RPV during mixed infections in the presence of MAV antiserum, however, *R. padi* was able to transmit BYDV-MAV and CYDV-RPV in the presence of BYDV-MAV antiserum (Rochow, 1970b). In the absence of antisera, *R. padi* was able to transmit CYDV-RPV for single infections and mixed infections of CYDV-RPV and BYDV-MAV, with one recorded single infection of BYDV-MAV. The outcomes from this study indicate that CYDV-RPV either wholly transcapsidated BYDV-MAV or substituted part of its capsid with CP of BYDV-MAV facilitating transmission, additionally that plants infected with both BYDV-MAV and CYDV-RPV had a more severe disease phenotype (Rochow, 1970b). This was further investigated in *A. byzantina* for BYDV-PAV and BYDV-MAV (Hu et al., 1988). Antibodies

for BYDV-MAV and BYDV-PAV were used to detect virus particles comprised of CP from both viruses, by testing mixed infection plants by coating with one antibody and trapping with another. By this technique virions, which had a capsid with subunits from both viruses were trapped and identified, and used in transmission tests with *R. padi*. The transmission tests showed that *R. padi* had a higher success rate of transmission of these virions compared to transmission of BYDV-MAV alone (Hu et al., 1988). This demonstrates that *R. padi* may be an efficient vector of BYDV-MAV when there is phenotypic mixing of BYDV-MAV by BYDV-PAV.

In a separate study, the presence of transcapsidation and phenotypic mixing was tested for BYDV-MAV, BYDV-PAV and CYDV-RPV and the vectors *R. padi* and *S. avenae* in oat (Creamer and Falk, 1990). Findings from this study demonstrated that during mixed infections of BYDV-MAV and CYDV-RPV, BYDV-MAV RNA is encapsidated by CP from CYDV-RPV and is vectored by *R. padi* able to transmit both viruses. Thus, BYDV-MAV had a one-way transcapsidation of CYDV-RPV CP, however a two-way transcapsidation system was observed for CYDV-RPV and BYDV-PAV enabling *S. avenae* to transmit CYDV-RPV successfully (Creamer and Falk, 1990). The results from this study showed the presence of two transcapsidation modes, one-way transcapsidation of BYDV-MAV by CYDV-RPV and two-way transcapsidation of BYDV-PAV and CYDV-RPV (Creamer and Falk, 1990). A comprehensive study was conducted for BYDV-MAV, BYDV-PAV, CYDV-RPV and CYDV-RMV (renamed *Maize yellow dwarf virus-RMV*, MYDV-RMV; Lefkowitz et al., 2017) using ELISA immunohybridization to trap transcapsidated and phenotypically mixed particles (Wen and Lister, 1991). Transcapsidation of BYDV-MAV by CYDV-RPV was shown to occur unidirectionally, the same was found for BYDV-PAV enclosed within the capsid of CYDV-RPV and the capsid of MYDV-RMV surrounding BYDV-MAV. A bidirectional transcapsidation was observed for BYDV-MAV and BYDV-PAV, as well as phenotypically mixed virus particles of BYDV-MAV and BYDV-PAV, and MYDV-RMV and CYDV-RPV (Wen and Lister, 1991).

These studies collectively show that transcapsidation and phenotypic mixing occur between the viruses which cause BYD disease, and they further suggest that mixed infections may be maintained in natural populations via this mechanism which enables the transmission of a virus by a non-vector aphid. The identification of both transcapsidation and phenotypic mixing in these studies needs to be validated by isolation and characterisation of virus particles which are comprised of mixed CP subunits, as well as studies on how viral RNA folds and interacts with a capsid comprised of another virus or with a mixed capsid.

## 1.6 Diversity of barley yellow dwarf virus

In Tunisia, studies have been conducted on outbreaks of BYDV in the largest production areas of barley, located in the north-east and western parts of the country (Najar et al., 2017). The studies provide an account of the outbreak in April 2012, in an effort to characterise the isolates present in the country using virus detection by tissue blot immuno-assay (TBIA) and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Findings from this study indicate that upon infection barley plants were symptomatic visibly showing signs of stunting and leaf discolouration, and with a higher disease prevalence in the north-east region. BYDV-PAV was most common with an incidence of ~64%, followed by BYDV-MAV (~16%) and CYDV-RPV (~3%). Sequence comparisons of the collected isolates to available GenBank accessions data revealed that the collected 6 Tunisian isolates analysed were most similar to isolates from Germany, BYDV-PAV AS1 (AJ810418) and the isolate BYDV-PAV 109 from the USA (EF521828). A phylogenetic analysis showed that BYDV-PAV collected from around the world may be divided into three clades, clade I is comprised of isolates from China, clade II contains three Chinese isolates and clade III contains a more varied mix of isolates from around the world – Japan, USA, Australia, Sweden, Germany, Iran, Pakistan and Tunisia (Najar et al., 2017). The close relationship between Tunisian isolates suggests that BYDV-PAV introduction may have occurred from a single source, most likely via the activity of a common vector to other clade III isolates, such as *R. padi*.

BYD disease has previously been identified as an important contributor to yield losses in grain cereals in Iran (Rastgou et al., 2005). Using DAS-ELISA BYDV-PAV was identified as the most prevalent virus causing disease, along with BYDV-MAV and CYDV-RPV (Rastgou et al., 2005). Phylogenetic analysis revealed that the BYDV-PAV isolate collected from infected Iranian wheat was most closely related to BYDV-PAV isolates from USA, Japan and France forming a distinct clade of isolates from around the world (Rastgou et al., 2005). CYDV-RPV diversity was assessed from a barley sample testing positive in DAS-ELISA, and subsequently used in phylogenetic analysis (Rastgou et al., 2005). Results from this analysis showed that the CYDV-RPV isolate collected from Iran was most related to a US isolate of CYDV-RPV from New York, and may be considered closely related to other worldwide isolates from Australia and Mexico (Rastgou et al., 2005). Information from this study indicated that Iranian BYD disease may be the result of common and widely distributed viruses of the family *Luteoviridae*. In addition, another study of BYD disease in 2005 - 2006 has shown the presence of BYDV-PAV, BYDV-MAV, BYDV-SGV and CYDV-RPV in Iran (Pakdel et al., 2010). The analysed Iranian BYDV-PAV isolates shared 92 – 97% nucleotide identity in ORF5 to isolates from Japan, USA and Australia. However, when considering ORF1, the Iranian BYDV-PAV isolates formed two distinct clades in a phylogenetic analysis: firstly a group containing isolates from Iran, USA, Australia and Japan and a second group of only isolates of central Iran, having 77 – 87% amino acid identity with isolates of the first group (Pakdel et al., 2010). Iranian BYDV-PAV isolates from different regions within the country shared a nucleotide identity of 84 – 86% (Pakdel et al., 2010).

Alaska, USA represents a geographically isolated region with cereal cultivation of barley and oat, but where B/CYDV infections occur (Robertson and French, 2007). DAS-ELISA and phylogenetic analysis of the CP region for BYD-associated viruses of Alaskan barley and oat identified BYDV-PAV, BYDV-PAS, CYDV-RPV and recombinant CYRDV-RPV as the main disease-causing agents (Robertson and French, 2007). Phylogenetic analysis revealed that BYDV isolates were separated into two distinct groups: isolates related to BYDV-PAS, showing 98% nucleotide identity to BYDV-PAS 129 from New York, USA and a second group of isolates more closely related to BYDV-PAV accessions from USA (99% nucleotide identity), Japan and Latvia (Robertson and French, 2007). Identified CYDV isolates formed three clades in a phylogenetic analysis: one clade with viruses having 98% nucleotide identity to CYDV-RPS, a polerovirus closely related to CYDV-RPV from Mexico; 99% nucleotide identity for a group of RPV isolates most similar to isolates from USA; a third group comprised recombinants between CYDV-RPV and CYDV-RPS, having recombination events within the CP region (Robertson and French, 2007). The results indicate that whilst Alaska has geographic isolation from other temperate cereal-growing regions, the B/CYDV isolates of Alaska are not distinct from described isolates from other parts of the world. The recombinant isolates of CYDV-RPS and CYDV-RPV indicate that these isolates may share a common vector species and that further understanding of the recombination event/s requires analysis of the vector species present and their ability to transmit virus.

B/CYDVs occur worldwide in many environments and are able to persist in areas which have limited number of cultivation cycles. The close relationships of isolates from different parts of the world indicate that B/CYDVs are successful in these environments and the genetic changes presently detected may reflect evolutionary processes which facilitate virus adaption to these new environments.

## 1.7 Barley yellow dwarf virus infection in grasses and cereals

BYD disease is globally present, occurring in differing environmental contexts, climatic conditions and under varied local and regional agricultural practices. BYD-associated viruses have been known to occur in both cultivated grasses as well as wild grass species, with many studies on the occurrence of infection in common wild grasses, symptom development and resistance. Added to this is a disease epidemiology driven by the presence of vector species, *R. padi* and *S. avenae*, which feed on wild grasses, from which virus may be

acquired and transmitted to cultivated crops. In this section, I will outline the diverse environments where B/CYDV is found, symptom development within these contexts and epidemiology of the virus.

### 1.7.1 Occurrence and infection in wild grass species

Owing to the large international agricultural sector, based around cereals, BYDV epidemiology in both wild and cultivated species has been studied extensively. A USA study conducted in the Midwest growing region, investigated the effect of BYDV infection on a common perennial pasture grass, switchgrass (*Panicum virgatum*) (Alexander et al., 2017). The aim of the assessment was to gain a deeper understanding of how cereal viruses such as BYDV, are harboured in native wild grasses, wild grasses symptomology, the effect of yearly environmental conditions and to use these results to develop a dataset for non-cultivated crops (Alexander et al., 2017). Switchgrass plants of varying localities were collected and subsequently grown in field conditions amongst invasive and natural competitor species, thereafter individuals were infected with BYDV-PAV and assessed during 2012 - 2014 recording symptom presence and effects on plant height, tiller formation, biomass, inflorescence formation and survival. The results from the assessment showed that BYDV-PAV-SHKR used for infection in 2012, had reduced detection in 2013 – 2014, however, naturally acquired BYDV-PAV and CYDV-RPV isolates were collected from plants. In respect to symptom development and effects on plant growth, it was shown that over the study period there was a significant reduction in aboveground biomass whilst below ground, the number of roots formed was similarly reduced. Further still, infected plants displayed typical symptoms with leaf discolouration, stunting and lower production of tillers as well as delayed flowering with lower seed set (Alexander et al., 2017). These effects were greatly influenced by annual environmental conditions supporting the notion that yearly conditions are an important factor in BYD disease outbreaks.

The US Pacific west coast is an area rich in indigenous grass species, as well as a productive area for cereals making it an ideal area for the studies on BYDV epidemiology in wild grasses and unravelling the relationship between disease in wild and cultivated areas (Ingwell et al., 2017). In 2010 - 2011, the Palouse Prairie area of Washington and Idaho was surveyed for BYDV infection, infected wild grass species and the relationship between managed and wild land (Ingwell et al., 2017). A total of 30 wild grass species were tested, 93% of which were positive for BYDV infection, whilst of the 2271 plants tested 46% were positive (Ingwell et al., 2017). This finding indicates that much of the grassland area is comprised of plants that may act as reservoirs for BYDV and that the abundance of BYDV within the area is spatially and temporally heterogeneous (Ingwell et al., 2017). Another important finding is that all infected grasses were asymptomatic for BYD disease, thus indicating that visual assessments are not reliable for disease identification in the wild (Ingwell et al., 2017). Viral diversity was low, with highest prevalence of single infections by BYDV-SGV and BYDV-PAV. An explanation of the low viral diversity may be linked to the diversity of vector species within the area, identifying the two aphid species *S. avenae* and *M. dirhodum* (Ingwell et al., 2017). When examining the relationship between grassland areas and cultivated land, it was found that with cereal farming intensification, the incidence of BYDV infection in wild grasses rose, most particularly farms in close proximity to wild areas had higher disease prevalence (Ingwell et al., 2017). These findings show that there is a large reservoir pool of BYDV in wild grasses, however, the exact nature of the interaction between isolates from wild grasses and cereal cultivation in this context remains unknown. In Florida State, USA, BYDV infection was studied in grasses commonly found during the summer period, such as bahiagrass (*Paspalum notatum*), limpgress (*Hemarthria altissima*) and eastern gamagrass (*Tripsacum dactyloides*), primarily to understand the potential of these as virus reservoirs (Hadi et al., 2012). Plant material was collected from the field in the summers of 2007 - 2008, from whole plants exhibiting symptoms of B/CYDV infection, and it was tested with enzyme-linked immunosorbent assay (ELISA) (Hadi et al., 2012). Bahiagrass and limpgress were found positive for BYDV-PAV and CYDV-RPV, while gamagrass was negative. In addition, aphid transmission tests showed that *R. padi* was unable to survive on bahiagrass indicating that this aphid is not the responsible vector for infection in bahiagrass and limpgress (Hadi et al., 2012). This study suggests that in Florida, infection of the wild grasses bahiagrass and limpgress occurs via

the activity of another aphid species, which may potentially act as a vector for virus transmission to cultivated barley, wheat and oat.

A study was conducted in autumn 2007 to investigate BYDV infection in varying landscapes in western France and determine the relationship between managed agricultural areas and natural grassland habitats, elucidate the epidemiology of BYDV in winter wheat, most especially to understand how grassland habitats act as sources for the virus (Gilabert et al., 2017). Wheat was grown in trays, placed in either wooded farmland and grassland habitat or in a managed area dominated by the cultivation of cereal crops (Gilabert et al., 2017). The assessment included: the colonization of *R. padi* on plants, the landscape from where aphids originated, incidence of BYDV-PAV infection and a spatio-temporal analysis of grassland and maize influence on virus occurrence. Findings outline that *R. padi* had the highest settlement on wheat seedlings, with an average aphid presence of 2% in both landscape conditions. *R. padi* aphids were further analysed and found to originate from C4-type plants, further supported by evidence that locations closest to cereals and maize had higher incidence of aphid visits (Gilabert et al., 2017).

These findings suggest that for adequate management of B/CYDV in cereal crops, grassland habitats require management to limit the number of host plants that act as reservoirs during the autumn period to ensure possible reduced transmission to crops.

#### 1.7.2 Infection on cultivated plant species: barley and wheat

B/CYDV infection in Europe has been well studied, with experimental data available from many countries. A study in the Czech Republic 2012 – 2015 investigated BYDV-PAV infection in winter wheat as well as in winter and spring barley varieties across locations and environmental conditions (Beoni et al., 2016). The assessment showed that BYDV-PAV infection is influenced by a yearly variation, such that between the study years the rate of infection in winter barley ranged from 0 – 70%, followed by reduced occurrence in winter wheat and was nearly negligible in spring barley. Further to this, it was found that the symptom development was consistent with literature and the most prominent visible feature changes were in leaf colouring and the reduced formation of tillers (Beoni et al., 2016). Comparisons between resistant and susceptible lines and cultivars of winter barley were able to categorise them based on the rate of infection. Symptom development was found to be influenced by the genetic background of the cultivar (Beoni et al., 2016). A 1995 – 1999 study investigating the occurrence of BYDV in Ukrainian winter wheat, found heterogeneous symptom formation within and between cultivars (Yuchimenko et al., 2001). Symptoms were consistent with previous studies: leaf reddening and yellowing, stunting and reduction in tiller formation as well as grain yields per tiller. Infection status for the study period was found to consistently range between 45 and 55% in tested plant material, however a severe disease outbreak was observed in 1998/1999 with an estimated 90% infection in winter wheat. BYDV-PAV, BYDV-MAV, BYDV-SGV and CYDV-RPV were identified via antibody assays of infected leaf material and aphid transmission tests (Yuchimenko et al., 2001).

BYD disease has long been studied in the USA where it has a large impact on the cultivation of cereals. One such assessment took place in Kansas, USA 2010 – 2012 in winter wheat sampled from both commercial and university trial fields (Rotenberg et al., 2016). A wide group of symptomatic and non-symptomatic wheat plants were assessed, with both susceptible and resistant varieties being scored for symptom development. Leaf samples were collected for TAS-ELISA to identify BYDV-PAV, CYDV-RPV and *Soil-borne wheat mosaic* virus as well as other wheat-infecting viruses and wheat tillers were collected to investigate effects on grain yields (Rotenberg et al., 2016). Several wheat-infecting viruses were found to infect winter wheat of Kansas, highest incidence was for BYDV-PAV: 37% in 2011 and 27% in 2012 found in both commercial and trial sites. BYDV-PAV was identified from both symptomatic and non-symptomatic leaf tissue. Estimations on tiller grain yield changes showed a reduced yield in BYDV-PAV infected plants compared to non-infected, 22% reduction in 2011 and 15% reduction in 2012 (Rotenberg et al., 2016).

Owing to the increased occurrence of BYDV in Brazil, a study on epidemiology of the virus was initiated to identify vector species and dynamics of vector populations preceding disease outbreaks (Parizoto et al., 2013). The study examined BYD disease throughout the summer and winter growing seasons of 2007 – 2009, in cultivars of winter wheat, oat (*A. strigose*) and summer maize (*Zea mays*) (Parizoto et al., 2013). Findings of this study indicate that *R. padi* (67% of total aphids) was the most abundant aphid species throughout the study period, having population highs in the mid-summer and mid-winter period. During this time a relationship between total highest number of aphids and viruliferous aphid population was observed, in which highest aphid numbers were recorded followed by a peak in viruliferous aphids. BYDV-PAV infection was recorded in 73% of oat, 53% wheat, and no B/CYDV infection was found in maize (Parizoto et al., 2013). Mixed infection of BYDV-PAV and BYDV-MAV was very low, found only in oat. BYDV infection was detected throughout the year, with lowest incidence of disease and viruliferous aphids coinciding during the change from winter to summer crop season (Parizoto et al., 2013). These findings provide insight on how BYDV persists, the presence of *R. padi* throughout the year may allow for it to act as reservoir with periodic infectious periods, during which favourable climate and host conditions facilitate disease outbreaks.

These studies cumulatively indicate that B/CYDV infection and severity is a function of the species under cultivation, the variety, developmental state of the individual plants and annual environmental conditions. It highlights that management of infection is reliant on an understanding of the regional setting, particularly identification of seasonal periods when plants are most vulnerable.

## 1.8 BYDV occurrence and infection in Sweden

Barley yellow dwarf disease has been identified as a high threat to Swedish agricultural production, thus studies have been undertaken to identify the occurrence, severity and incidence in important cereal crops. One such investigation sought to elucidate infection in oat (“Sol II” variety) and barley as well as grass species, which were hypothesised to act as viral repositories and thus as a source of inoculum (Eweida and Rydén, 1984). This study was conducted during the summer of 1982 using both field collection of grasses and cereals, followed by greenhouse inoculation of oat and barley with field-collected BYDV isolates (Eweida and Rydén, 1984). Grass species infected with the isolate BYDV 39/78 (severe) highlighted the variability in symptom development and aphid transmission efficiency: species showing light to moderate stunting were *Agrostis stolonifera*, *Festuca ovina*, *F. pratensis*, *Lolium perenne* and *Poa trivialis* (Eweida and Rydén, 1984). The aphid transmission efficiency of BYDV was also higher for these plants compared to plants which had no symptoms. The most severe symptom occurred in *Cynosurus critatus* plants, which were severely stunted or died (Eweida and Rydén, 1984). The inoculum of 25 BYDV isolates was used to infect oat and barley using *R. padi* and *S. avenae* (Eweida and Rydén, 1984). The pathogenicity of each isolate was characterised and the transmission ability of vectors evaluated; in which it was found that 20 of 25 isolates studied were transmitted exclusively by *R. padi* and three isolates by *S. avenae* (Eweida and Rydén, 1984). Further still, the study showed that for 14 isolates, infection resulted in severe symptoms and the majority of the virus isolates were transmitted by *R. padi* (Eweida and Rydén, 1984). The results from the above assessment indicate that BYDV detection based on symptom development in wild grasses is not reliable.

A more recent assessment of BYDV diversity was conducted in Sweden and Latvia during 2000 – 2001 with samples of both grass species, *L. perenne* and *Poa sp.*, as well as of cultivated barley and oat (Bisnieks et al., 2004). The findings indicated that collected grasses displayed no symptom development whilst cultivated barley and oat from Latvia and Sweden showed signs of leaf yellowing and stunting (Bisnieks et al., 2004). Further still, sequence analyses of the CP gene indicate that Latvian and Swedish BYDV-PAV isolates were very closely related, having shared nucleotide identities of 93 – 99%, with BYDV-MAV isolates from these countries showing a similar relationship (Bisnieks et al., 2004). When comparing the Latvian and Swedish isolates to those present in databases, it becomes clear that the BYDV-MAV isolates were most closely related to isolates from USA and China, which may be because the vector *S. avenae* of BYDV-MAV

is globally distributed (Bisnieks et al., 2004). When investigating BYDV-PAV diversity, isolates were more varied falling into two distinct groups: isolates infecting grasses and those infecting oat, whilst isolates infecting barley were present in both groups (Bisnieks et al., 2004). In addition, BYDV-PAV “PAV-Sal1” was identified as a unique and distinct isolate from Latvia, and was thus suggested as representing a new tentative species, BYDV-OYV (Bisnieks et al., 2004).

Oat is an economically important crop in Sweden, and thus the effect of BYDV-PAV infection at differing developmental stages was studied in May - August 2002 (Bisnieks et al., 2005). Oat cultivar “Stork” was evaluated for symptom development and measurement of biomass upon infection of a BYDV-PAV isolate (PAV-Sto) collected from Uppsala, Sweden (Bisnieks et al., 2005). Plants infected at an early growth stage had more severe symptoms with reduced plant height, reddening of leaf tips, lower production of tillers per plant and unfilled kernels. Thus, it can be said that infection at an earlier developmental stage results in reductions in grain mass and dry biomass (Bisnieks et al., 2005). Cumulatively the results from this experiment show that the age of plants upon infection affects the severity of BYD symptom development (Bisnieks et al., 2005). Further still it highlights the need to assess the effects of infection by individual BYDV isolates on agronomically important aspects of plant development (Bisnieks et al., 2005).

## 1.9 Thesis projects

In the spring of 2015 and 2017, BYD disease was observed to affect cereals in the southern agricultural region of Sweden as well as Denmark. As BYD disease has previously been studied in Sweden (Bisnieks et al., 2004, 2006; Eweida and Rydén, 1984) and the predominant cause of infection identified as BYDV, a primary aim of this thesis was:

1. Investigate the diversity of BYDV and CYDV isolates in Swedish winter cereals during disease outbreaks in spring 2015 and 2017

As BYDV and CYDV have been experimentally demonstrated to show phenotypic mixing and transcapsidation during mixed infections, and the disease outbreaks of 2015 and 2017 provide naturally infected plant material in which such a system may be investigated, thus a second aim was to:

2. Investigate the presence of transcapsidation or phenotypic mixing in plants infected by BYDV and CYDV

The results of this project will be used in assisting Swedish agricultural agencies and farmers with information for management of BYD disease and provide further scientific insight on BYDV and CYDV infection and transmission in cultivated cereals.

## 2 Methods and Materials

### 2.1 Plant Material

Symptomatic plants of winter barley and winter wheat were collected during a BYD outbreak in spring 2015. In May 2017, symptomatic plants of winter barley and winter rye (*S. cereale*) were collected. All samples were from farms and fields in the counties of Skåne and Östergötland, southern Sweden (Table 1).



*Figure 2.* Field with barley yellow dwarf disease in Krageholm, Skåne, Sweden. Visible are symptoms of leaf yellowing throughout the field. Photo: Christer Pålsson

Table 1. Plant material for analysis of BYDV and CYDV.

Year		Location		Species	Cultivar	Sample ID		
2015	April	County of Skåne	Klagstorp	Winter barley ( <i>H. vulgare</i> )	Unknown	13		
			Dybäck		Unknown	14		
			Hasslarp		Agropos	15		
			Simrishamn		Unkown	16a		
			Simrishamn		Unknown	16b		
			Åstorp		Unknown	17		
			Arendala		Unknown	18		
			Trolleberg		Unknown	19		
			Brenestad		Unknown	40		
			Unknown		Zoom	41		
			Brösarp		Unknown	43		
					Slimminge	Winter wheat ( <i>T. aestivum</i> )	Maribos	38
					Krageholm		Unknown	39
		May		Östra Vemmenhög		Ellvis	36	
			Slimminge		Praktik	37		
2017	May	County of Östergötland	Vadstena	Winter barley ( <i>H. vulgare</i> )	Sy Wootan	RoLM7		
			Vadstena		Sy Wootan	RoLM10		
			Vadstena		Sy Wootan	RoLM11		
			Vadstena		Sy Wootan	RKM2		
			Vadstena		Sy Wootan	RKM4		
			Klosterstad		Mercurio	RKM6		
			Klosterstad		Mercurio	RKM7		
			Klosterstad		Mercurio	RKM10		
			Ödeshög		Sy Wootan	FM2		
			Ödeshög		Sy Wootan	FM5		
			Ödeshög		Sy Wootan	FM10		
					County of Skåne	Bjuv	Unknown	GM6
						Bjuv	Unknown	GM9
			Bjuv	Unknown	GM15			
		Äspö	Winter rye ( <i>S. cereale</i> )	Unknown	WR1			
		Äspö		Unknown	WR10			
		Äspö		Unknown	WR11			

## 2.2 DAS-ELISA and Viral RNA Release

### 2.2.1 DAS-ELISA

DAS-ELISA of BYDV-PAV, BYDV-MAV and CYDV-RPV was conducted on leaf samples of winter barley and winter rye collected from fields in spring 2017. Samples collected in 2015 were tested by DAS-ELISA at the time of their collection. All samples were tested at the Department of Plant Biology, Swedish University of Agricultural Sciences using DAS-ELISA kits and protocols developed by BIOREBA (BIOREBA, 2017a). All buffers were freshly prepared before the start of the assays. Leaf samples were ground and homogenized in extraction buffer and stored at -20°C. 96-well plates were coated with 200 µl of antibody against either BYDV-PAV, BYDV-MAV or CYDV-RPV, wrapped in parafilm and stored at 4°C overnight. Prepared wells were washed 3 – 4 times in washing buffer, allowed to dry and thereafter 200 µl of sample homogenate was added per well. Each sample had a double replicate along with both a positive control derived from infected plant material and negative control from uninfected plants (BIOREBA, 2017b) and blank (H<sub>2</sub>O). Plates were wrapped tightly in parafilm and incubated in the dark overnight at 4°C. Plates were washed 3-4 times in washing buffer and allowed to dry, followed by the addition of 200 µl conjugate buffer and incubation in the dark for 2 hr at 37°C. The washing procedure was then repeated once more. pNPP was dissolved in substrate buffer just before use and 200 µl was added per well. The plates were then incubated in the dark at room temperature for 1 hr, and the absorbance was read spectrophotometrically at 405 nm.

### 2.2.2 Viral RNA Release

After reading of plates spectrophotometrically, virus release was initiated using the method described by Harju et al. (2005). Virus Release Buffer (VRB) was freshly prepared from 10 mM Tris-HCl (pH 8.0) and Triton X-100 (1% v/v). To each well in 96-well plates, 50 µl of VRB was added. The plates were covered and allowed to shake at 65°C for 5 minutes (Harju et al., 2005). Thereafter, the RNA concentration was determined and the released RNA stored at -20°C to be used in reverse transcription and PCR.

## 2.3 RNA Extraction and RT-PCR

### 2.3.1 RNA Extraction

RNA was extracted according to the protocol by Oñate-Sánchez and Vicente-Carbajosa (2008). Discoloured leaves were ground in cell lysis solution. Homogenized material was decanted into eppendorf tubes and incubated for 5 minutes at room temperature. 200 µl of protein-DNA precipitate solution was added to the homogenate. The tubes were gently inverted and incubated for 10 minutes at 4°C and subsequently centrifuged at 4°C for 10 minutes. The supernatant was transferred to clean eppendorf tubes where 600 µl of iso-propanol was added. The tubes were inverted and centrifuged for a further 4 minutes and kept overnight at -20°C. The supernatant was removed, and the pellet was washed in 1 ml 70% ethanol, air-dried at room temperature for 1 hour and resuspended in 50 µl RNase-free H<sub>2</sub>O.

### 2.3.2 Reverse transcription and PCR

The cDNA synthesis by reverse transcription (RT) was conducted as described by Malmstrom and Shu (2004), using the universal Yan-R primer followed by multiplex PCR using the primers: Yan-R, Shu F, S2a Forward and S2b Forward (Table 2). For RT, a master mix solution (10 mM dNTP, Yan-R primer and H<sub>2</sub>O) was prepared for all samples (total reaction volume: 15 µl). The reaction was heated at 65°C for minutes and placed on ice. The following mixture was added to each tube: 4 µl 5x BF, 0.5 µl RNase, 0.5 µl Revert Aid

reverse transcriptase (Thermo Scientific) for a total reaction volume of 20  $\mu$ l. Synthesis of cDNA was carried out at 56°C for 1 hr and the reaction stopped by incubation at 70 °C for 10 minutes.

Table 2. Primers used for multiplex RT-PCR to determine BYDV-PAV, BYDV-MAV and CYDV-RPV infection in barley, wheat and rye. Protocol and primers used are derived from Malmstrom & Shu (2004).

Primer	Sequence (5' - 3')
Yan-R	TGTTGAGGAGTCTACCTATTTG
Shu-F	TACGGTAAGTGCCCAACTCC
S2a-F	TCACCTTCGGGGCGTCTCTATCAG
S2b-F	TCACCTTCGGGGCGTCTCTTTCTG

For multiplexed PCR, each reaction contained: 1  $\mu$ l of 10 mM dNTP, 1  $\mu$ l of 20  $\mu$ M Shu F primer, 1.5  $\mu$ l of 20  $\mu$ M Yan R primer, 0.25  $\mu$ l of 20  $\mu$ M S2a forward primer, 0.25  $\mu$ l of 20  $\mu$ M S2b forward primer, 10 x Reaction Buffer, 0.5  $\mu$ l Dream Taq DNA polymerase (Thermo Scientific) and 11.5  $\mu$ l H<sub>2</sub>O for a volume of 18  $\mu$ l to which 2  $\mu$ l of cDNA was added for a total reaction volume of 20  $\mu$ l. The PCR products were run on a 1% agarose gel electrophoresis along with 1kb DNA ladder. Thereafter the DNA products were viewed and photographed in UV light, cut out from the gel, weighed and stored at -20°C.

## 2.4 Cloning and Transformation

DNA products were purified from 1% agarose gel using the GeneJet Gel Extraction Kit (Thermo Scientific) and following the manufacturer's protocol. The concentration of eluted DNA was measured via NanoDrop (Thermo Scientific) and thereafter stored at -20°C. DNA was subsequently cloned using the CloneJet PCR Cloning Kit (Thermo Scientific), as per the sticky end cloning protocol into Pjet1.2 plasmid vector. The NEB®10 transformation protocol was used to transform plasmids into DH5 $\alpha$  competent *E. coli* cells. Cells were grown for 1 hr at 37°C in LB media with shaking at 200 rpm, and thereafter 100  $\mu$ l of cells were plated on antibiotic selective media containing 100 mg/ $\mu$ l ampicillin. Transformed bacteria were grown overnight in the dark at 37°C and plasmid DNA was isolated and analysed by restriction digests using BglII. Plasmids with inserts of the expected size were sent for sequencing to Macrogen.

## 2.5 Sequencing and phylogenetic analysis

A total of 42 clones were sent to Macrogen for sequencing. Sequence data was analysed in Lasergene DNASTar to remove forward and reverse primers. Trimmed sequence data was used in the comparative sequence alignment tool, Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide sequence data of the cloned amplification products was used in BLASTn, an algorithm which compares input nucleotide data to that of nucleotide sequences already present in the GenBank database and outputs the most probable identity of input data and the region(s) of most similarity (McGinnis and Madden, 2004).

Sequence data was further used in a phylogenetic analysis program, Molecular Evolutionary Genetics Analysis 7 (MEGA 7) (Kumar et al., 2016). Trimmed input nucleotide sequence data, 789 nt length, was used for multiple sequence alignment (MSA) using the CLUSTAL W (Thompson et al., 1994) algorithm. Aligned sequence data was checked to ensure that all sequences were in the same direction and erroneous nucleotide calls were checked in sequence chromatograms. The MSA were used for phylogenetic analysis using the Maximum Likelihood (ML) algorithm to infer relationships between isolates, in which the most frequently recurring relationships are reported. This was further tested using bootstrap analysis (Felsenstein,

1985), for 500 iterations of tree computations to output the consensus tree. Trimmed sequence data was also input in DNASTar MegAlign, a CLUSTAL W MSA program for determining nucleotide identities. The CP nucleotide sequences of BYDV isolates collected in 2015 and 2017 were analysed together with available GenBank accessions for BYDV (Table 3).

Table 3. *Origin and description of BYDV isolates used for phylogenetic analysis*

Accession No.	Name	Host Plant	Country
AF218798	BYDV-PAS PAV129	Oat	USA
AJ563410	BYDV-PAV-Saldus1	Oat	Latvia
AJ563411	BYDV-PAV-Saldus2	Oat	Latvia
AJ563412	BYDV-PAV-Storvreta	Barley	Sweden
AJ563413	BYDV-PAV-Priekuli1	Oat	Latvia
AJ563414	BYDV-PAV-Priekuli2	<i>Lolium perenne</i>	Latvia
AJ563415	BYDV-PAV-Sweden	<i>Poa</i> sp.	Sweden
AJ563416	BYDV-MAV-Storvreta1	Barley	Sweden
AJ563417	BYDV-MAV-Storvreta2	Barley	Sweden
AJ563418	BYDV-MAV-Priekuli	Barley	Latvia
AJ563419	BYDV-MAV-Jelgava	Barley	Latvia
AJ810418	BYDV-PAV ASL1	Barley	Germany
D85783	BYDV-PAV JPN-PAV	Barley	Japan
DQ079609	BYDV-PAV PAV-Express	Wheat	France
DQ079611	BYDV-PAV PAV-Zhong	Wheat	France
DQ631855	BYDV-PAV Tehama- 258	<i>Elymus multisetus</i>	France
EF521828	BYDV-PAV 0109	Barley	USA
EF521829	BYDV-PAV 005-PAV	<i>Bromus secalinus</i>	USA
EF521841	BYDV-PAV 052		Sweden
EF521843	BYDV-PAV 0100		USA
EU332322	BYDV-PAV 05ZZ6	Wheat	China
EU332323	BYDV-PAV 05ZZ7	Wheat	China
EU332324	BYDV-PAV 05ZZ9	Wheat	China
EU332330	BYDV-PAV 06JN2	Wheat	China
EU402390	BYDV-GAV 05WN5	Wheat	China
KP771878	BYDV-PAV Kerman	Barley	Iran
KT252975	BYDV-PAV Chk04	Wheat	Pakistan
KT252976	BYDV-PAV DI144	Wheat	Pakistan
KU170668	BYDV-PAV-KS-SHKR	Wheat cv. Shocker	USA

## 3 Results

### 3.1 DAS-ELISA of winter cereals collected in spring 2015 and 2017

DAS-ELISA was conducted on a total of 11 barley and 4 wheat plants collected in April and May 2015 and 15 barley and 3 rye samples collected in May 2017, testing for the presence of serotypes BYDV-MAV, BYDV-PAV and CYDV-RPV (Figure 3).

During the disease outbreak of 2015, DAS-ELISA showed that BYDV-MAV and BYDV-PAV were the two most common serotypes in wheat and barley (Table 4). BYDV-PAV and BYDV-MAV were detected in 9 of 11 (81%) barley samples, and all four (100%) wheat samples (Table 4). All samples positive for BYDV-PAV were also positive for BYDV-MAV, there were no recorded single infections of either virus (Table 4). CYDV-RPV was detected less frequently, identified in 3 of 11 (27%) barley samples tested and 2 of 4 (50%) of wheat samples (Table 4). There were triple infections of BYDV-PAV, BYDV-MAV and CYDV-RPV for 3 of 11 (27%) barley samples and 2 of 4 (50%) wheat samples. No single CYDV-RPV infection or double infection of CYDV-RPV with either BYDV-PAV or BYDV-MAV was detected.

In the disease outbreak of 2017, DAS-ELISA showed that BYDV-MAV was the most common serotype infecting wheat and barley (Table 5). BYDV-MAV was detected in 14 of 15 (93%) barley plants and all three (100%) rye plants (Table 5). BYDV-PAV was present in 6 of 15 (40%) barley plants tested and CYDV-RPV in 2 of 15 (13%) barley plants tested (Table 5). Samples were collected from symptomatic plants, however, rye did not have as visible symptoms of BYDV or CYDV infection, whilst all barley plant samples showed symptoms of infection during the 2017 outbreak. All rye samples were positive for BYDV-MAV, with one sample also being positive for BYDV-PAV. No CYDV-RPV infection was detected among the rye samples (Table 5).

DAS-ELISA tested plants from the 2015 and 2017 BYD outbreaks were further used for PCR and sequencing to identify the BYD-associated viruses present.

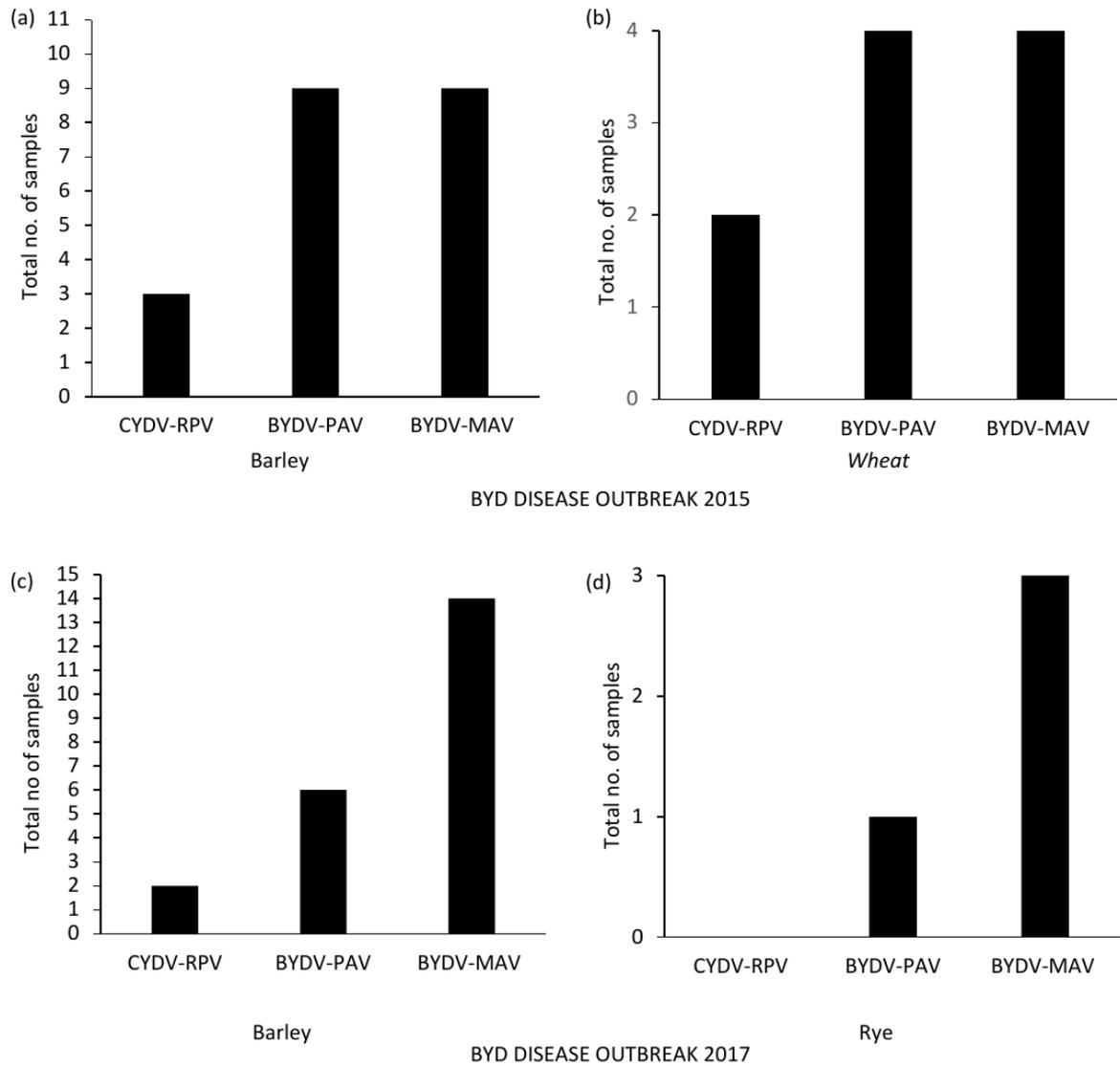


Figure 3. DAS-ELISA results for plants collected during disease outbreaks in April and May 2015 and 2017 in Skåne and Östergötland, Sweden. (a) Barley plants from 2015 were positive for BYDV-PAV and BYDV-MAV, whilst a low number were positive for CYDV-RPV (b) All wheat plants from 2015 were positive for both BYDV-PAV and BYDV-MAV, and half of the plants were positive also for CYDV-RPV. (c) Barley plants from 2017 were positive for BYDV-MAV, BYDV-PAV and a few for CYDV-RPV. (d) Rye plants were positive for BYDV-MAV and BYDV-PAV, whilst none were positive for CYDV-RPV.

Table 4. DAS-ELISA of B/CYDV-infected wheat and barley samples collected from Skåne in April and May 2015

Species	Sample ID	CYDV-RPV	BYDV-PAV	BYDV-MAV
Winter barley ( <i>H. vulgare</i> )	13	-	+	+
	14	-	+	+
	15	-	+	+
	16a	-	+	+
	16b	-	-	-
	17	-	+	+
	18	-	-	-
	19	-	+	+
	40	+	+	+
	41	+	+	+
	43	+	+	+
Winter wheat ( <i>T. aestivum</i> )	36	+	+	+
	37	+	+	+
	38	-	+	+
	39	-	+	+

Positive detection of infection is indicated with "+", negative detection "-"

Table 5. DAS-ELISA of B/CYDV-infected barley and rye collected from Skåne and Östergötland in May 2017

Species	Sample ID	Mean DAS-ELISA Absorbance (405nm), n= 2		
		CYDV-RPV	BYDV-PAV	BYDV-MAV
	Negative Control	0.09	0.15	0.09
	Positive Control	1.36	0.13	1.00
Winter barley ( <i>H. vulgare</i> )	RoLM 7	0.15	0.29	0.79*
	RoLM 10	0.16	0.28	0.49*
	RoLM 6	0.14	0.3*	0.64*
	RKM 6	0.18*	0.28	0.88*
	RKM 4	0.11	0.33*	0.7*
	RKM 2	0.12	0.22	0.48*
	RKM 7	0.15	0.27	1.18*
	RKM 1	0.14	0.25	0.79*
	RKM 10	0.13	0.34*	0.59*
	FM 5	0.14	0.33*	0.49*
	FM 2	0.14	0.4*	0.76*
	FM 10	0.14	0.19	0.69*
	GM 6	0.16	0.25	0.17
	GM 9	0.15	0.28	0.63*
	GM 15	0.51*	2.89*	0.49*
Winter rye ( <i>S. cereale</i> )	WR 1	0.16	0.25	0.46*
	WR 10	0.11	0.31*	0.55*
	WR 11	0.10	0.20	0.55*

Positive detection of infection is double the value of negative control and indicated with \*

### 3.2 Reverse transcription and multiplex PCR

RNA extractions from B/CYDV-infected plant material collected in 2015 were used for reverse transcription and multiplex PCR to confirm the infection (Malmstrom and Shu, 2004). The RNA concentration after extraction from samples of barley and wheat was high and the RNA was found to be pure, except for sample 36 (Table 6). The PCR results show two distinctive bands, with one of ~ 800 bp which is for BYDVs and one of ~250 – 500 bp for CYDVs (Figure 4). Samples which were positive for BYDV were: 13, 14 15, 18A, 36, 37, 38, 39 and 43. Samples 16A and 40 were positive for both BYDV and CYDV, whilst sample 41 was negative for B/CYDV (Figure 4).

Table 6. Concentration of RNA extracted from samples of barley and wheat collected in spring 2015

Species	Sample ID	RNA (ng)	260/280
Winter barley ( <i>H. vulgare</i> )	13	581.6	1.67
	14	907.2	1.67
	15	5674.6	1.72
	16a	42.2	1.84
	16b	838	1.73
	17	3809	1.77
	18	1767	1.72
	19	1332.8	1.8
Winter wheat ( <i>T. aestivum</i> )	36	2.2	1.1
	37	4020.8	1.78
	38	1197.4	1.77
	39	6217.8	1.77
Winter barley ( <i>H. vulgare</i> )	40	1857.2	1.8
	41	941.8	1.77
	43	2015.6	1.8



Figure 4. PCR products of B/CYDV-infected samples collected in April and May 2015. 800 bp band corresponds to BYDVs, whilst a band at 250-500 bp corresponds to CYDVs. 1 kb DNA ladder is "L".

Upon completion of DAS-ELISA of BYDV-MAV and CYDV-RPV for samples from spring 2017, VRB was applied to initiate release of captured virion RNA (Harju et al., 2005). The released RNA is enriched with viral RNA compared to conventional RNA extracts from leaves (Oñate-Sánchez and Vicente-Carbajosa, 2008). The concentration was high for RNA released from BYDV-MAV DAS-ELISA, except for sample RKM1 (Table 7). Viral RNA release from CYDV-RPV DAS-ELISA succeeded for 4 of 18 samples (Table 7). Released RNA was used in RT-PCR (Malmstrom and Shu, 2004) with samples RoLM6, RKM4, RKM2, RKM1, FM5 and FM10 showing two distinctive bands in the gel, designated “A” and “B” corresponding to sizes consistent with BYDVs at ~800 bp and CYDVs at ~250 – 500 bp. The DNA concentrations of purified RT-PCR products were low, at less than 10 ng/μl (Table 8). There were low DNA concentrations or erroneous 260/280 results, an indicator of DNA purity, for samples FM2A, FM2B and FM10A. RT-PCR of released RNA from CYDV-RPV DAS-ELISA was not successful. Therefore, samples prepared from DAS-ELISA viral RNA release were not used for further analysis, and instead leaf samples from 2017 BYDV infected winter barley were used in conventional RNA extraction followed by RT-PCR, cloning and sequencing. A minimum of 1 clone per sample after successful RT-PCR was prepared and sent for sequencing (Table 9). Four clones were prepared each for samples 13 and 14 of estimated size ~800bp based on gel electrophoresis, sample 16a had 2 clones each prepared of sizes ~250-500 bp and ~800 bp from 2015 and sample RKM2 from 2017 had 3 clones of estimated size ~250-500 bp and 2 clones of ~ 800 bp (Table 9).

Table 7. Concentration of released RNA from BYDV-MAV and CYDV-RPV DAS-ELISA of spring 2017 barley, wheat and rye

	Sample ID	RNA (ng/μl)
BYDV-MAV VRB	RoLM7	416.8
	RoLM6	513.16
	RoLM10	383.94
	RKM6	581.96
	RKM4	455.95
	RKM7	326.78
	RKM1	
	RKM2	702.09
	FM5	438.81
	FM2	534.33
	FM10	458.23
	GM6	571.58
	GM9	646.75
	GM15	169.46
	WR1	402.56
	WR10	542.54
WR11	213.99	
CYDV-RPV VRB	GM15	422.17
	RKM1	1097.12
	RKM10	659.8
	RKM7	404.3

Table 8. Concentration of DNA after RT-PCR from released RNA of BYDV-MAV DAS-ELISA of spring 2017 barley and wheat

	Sample ID	DNA (ng/μl)	260/280
BYDV-MAV VRB	RoLM7	5.68	1.27
	RoLM10	4.26	1.08
	RoLM6A	3.23	2.58
	RoLM6B	3.67	1.52
	RKM4A	2.18	1.79
	RKM4B	3.56	1.33
	RKM2A	6.51	1.77
	RKM2B	6.86	1.98
	RKM7A	6.85	2.19
	RKM7B	6.54	1.93
	RKM1A	3.62	1.63
	RKM1B	6.93	8.09
	RKM10	6.41	9.01
	FM5A	4.29	2.37
	FM5B	0.98	1.49
	FM2A	1.07	-9.24
	FM2B	-1.16	0.61
	FM10A	1.05	-2.41
	FM10B	2.81	1.56

Table 9. Number of clones per sample sequenced for BYDV disease outbreaks in 2015 and 2017

Year	Sample ID	No. of Clones	
		PCR Product Size *	
		~250-500bp "CYDV"	~800bp "BYDV"
2015	13		4
	14		4
	15		2
	16a	2	2
	18		2
	19		3
	37		1
	38		1
	40	1	1
	43		2
2017	RoLM6		2
	RoLM7		3
	RKM1		1
	RKM2	3	2
	RKM7	1	2
	FM5		2

\*PCR product size as determined by gel electrophoresis band size compared to 1kb DNA ladder.

### 3.3 Analysis of the BYDV coat protein gene sequence

The 2015 (24 clones) and 2017 (16 clones) sequence data was analysed with BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and found to have 95 – 99% identity to BYDV-PAV and BYDV-PAS isolates, save for one identified as Barley virus G (BVG). Two clones, 16a-2 and 16a-3 had highest identity to BYDV-PAV isolates whilst other clones had highest identity to BYDV-PAS or BYDV-PAV isolates which should be classified as BYDV-PAS. BYDV-PAV isolates identified with 97–99% identity were BYDV-PAV-KS-SHKR (KU170668), BYDV-PAV 0109 (EF521828) and BYDV-PAV ASL1 (AJ810418). The two former isolates were from wheat and barley respectively, in the mid-west USA, whilst the latter isolate was from barley in Germany. Among the isolates classified as BYDV-PAS, the isolates from southern Sweden showed highest identity at 95-96% to BYDV-PAS-PAV 129 (AF218798) from the USA, and three isolates identified in New Zealand BYDV-PAS PAS Tcb4-1 (EF408154) isolated from *Dichelachne crinita*, BYDV-PAS PAS Wpc1 (EF408155) from wheat and BYDV-PAS PAS waw5-1 (EF408152) from *Microlaena stipoides*. Other identified sequences were BYDV-PAV Tehama 258 (DQ631855) isolated from *Elymus multisetus* in western USA and BYDV-PAV 064 (EF521850) from oat in Alaska, USA.

Clones 16a-2 and 16a-3 shared 99.6% identity with BYDV-PAV isolates from New Zealand, France, USA, China and Sweden. Identified BYDV-PAV isolates were, BYDV-PAV PAV T22H3 (EF408176) isolated from *Poa cita* in New Zealand, and four French isolates from wheat BYDV-PAV PAV TC14 (DQ079612), BYDV-PAV Zhong (DQ079611), BYDV-PAV Sunstar (DQ079610.1) and BYDV-PAV Express (DQ079609). Two isolates were identified from *Avena fatua* in USA, BYDV-PAV YOLO 274 (DQ631850) and BYDV-PAV YOLO 270 (DQ631848), two isolates from wheat in China, BYDV-PAV 06KM14 (EU332332) and BYDV-PAV 05GG2 (EU332309), and BYDV-PAV 052 (EF521841) from Sweden.

Isolate 40 CYDV (333nt), isolated from barley in Skåne identified as matching BVG from South Korea with an identity of 99.4% to the following isolates: BVG Jeju (LC159486) isolated from *Setaria italica*; two isolates from *Panicum miliaceum*, BVG Uiseong (LC259081) and BVG Uisung (LC159487); BVG Gimje (KT962089) isolated from barley.

BLASTn sequence analysis did not identify BYDV-MAV despite positive DAS-ELISA results for barley and wheat from spring 2015, and barley and rye in 2017. CYDV-RPV was not identified by BLASTn even if positive by DAS-ELISA in barley and wheat from spring 2015, and in barley in 2017.

### 3.4 Phylogenetic analyses of BYDV infection in southern Sweden 2015, 2017

The nucleotide identities between isolates collected during disease outbreaks in 2015 and 2017 were examined using pairwise nucleotide alignment of the 789 nt CP region (Figure 5). Nucleotide identities (%) between isolates from outbreaks in 2015 and 2017 show that most of these isolates share >99% identity, irrespective of the plant host species or year of collection (Figure 5a). Two clones, 16a-2 and 16a-3 of sample 16a collected from barley during the 2015 disease outbreak were divergent and showed lower identity compared to the other isolates, at 90% for 16a-3 and 96% for 16a-2 (Figure 5a). When further analysed by maximum likelihood phylogenetic analysis, the close relationship of isolates irrespective of year collected and host species was confirmed. All sequences, except 16a-2 and 16a-3, formed a well-supported group (bootstrap value: 99%) (Figure 5b). As previously indicated by the pairwise nucleotide analysis, clones 16a-2 and 16a-3 are distinct and separate from one another (Figure 5b).

To better contextualize the relationship(s) of virus isolates in the 2015 and 2017 disease outbreaks with other BYDV isolates globally, the sequences were analysed with those of previously studied isolates from USA, Germany, Pakistan, France, Iran, China, Japan and Sweden (Figure 6). Isolates from USA showing

high identity (99%) to Swedish BYDV isolates collected in 2015 and 2017 were BYDV-PAV KS SHKR (KUI70668) and BYDV-PAV 0109 (EF521828) from wheat and barley (Figure 6a). These isolates along with German isolate BYDV-PAV ASL1 (AJ810418) formed part of a larger well-supported group (bootstrap value: 95%) in the phylogenetic analysis (Figure 6b). This group represents BYDV-PAS, wherein some BYDV-PAV isolates in GenBank have probably been incorrectly classified and should be BYDV-PAS. The sequence of clone 16a-3 grouped among the BYDV-PAV isolates collected globally from Sweden, France, USA, Japan, Iran and Pakistan (Figure 6b). The nucleotide identity of 16a-3 was 97 – 99% to these isolates (Figure 6a). In the tree, 16a-2 was located between the BYDV-PAS group of isolates and the BYDV-PAV isolates (Figure 6b). This indicates that it has an intermediary sequence structure of BYDV-PAV and BYDV-PAS. Pairwise nucleotide (%) identities of isolates indicate that the included isolates from China shared a lower level of identity (77-87%) with the Swedish isolates from 2015 and 2017 (Figure 6a).

The sequences of Swedish BYDV isolates of 2015 and 2017 were further analysed together with those of previously recorded Swedish and Latvian BYDV-PAV and BYDV-MAV isolates (502 nt) (Bisnieks et al., 2004) (Figure 7). Isolates were analysed separately from those in Figure 6, as the GenBank accessions are 502 nt in length and do not completely overlap in the CP region with sequences determined in this study. It was observed that the isolates of the disease outbreaks in 2015 and 2017 maintain a well-supported grouping (bootstrap value: 98%) (Figure 7b), as the previously described BYDV-PAS grouping (Figure 6a). Nucleotide identities for these isolates were well >98% indicating that the isolates from these outbreaks are closely related (Figure 7a). It is also clearly observed that clones 16a-2 and 16a-3 were separated from this larger group, in which 16a-3 grouped with both Swedish and Latvian isolates of BYDV-PAV (bootstrap value: 77%) (Figure 7b). The sequence of clone 16a-2 laid between these two groupings further supporting the notion of an intermediary sequence structure (Figure 7b). Maximum likelihood phylogenetic analysis of shorter amplified PCR products, isolates 16a-CYDV (243 nt), 16a-4 CYDV (242 nt), FM5B-6 (242 nt), RKM1B-1 (429 nt) and 40 CYDV (333 nt) indicated that isolate 40 CYDV, identified as BVG by BLASTn analysis, is separate and distinct from other isolates (Figure 7c).

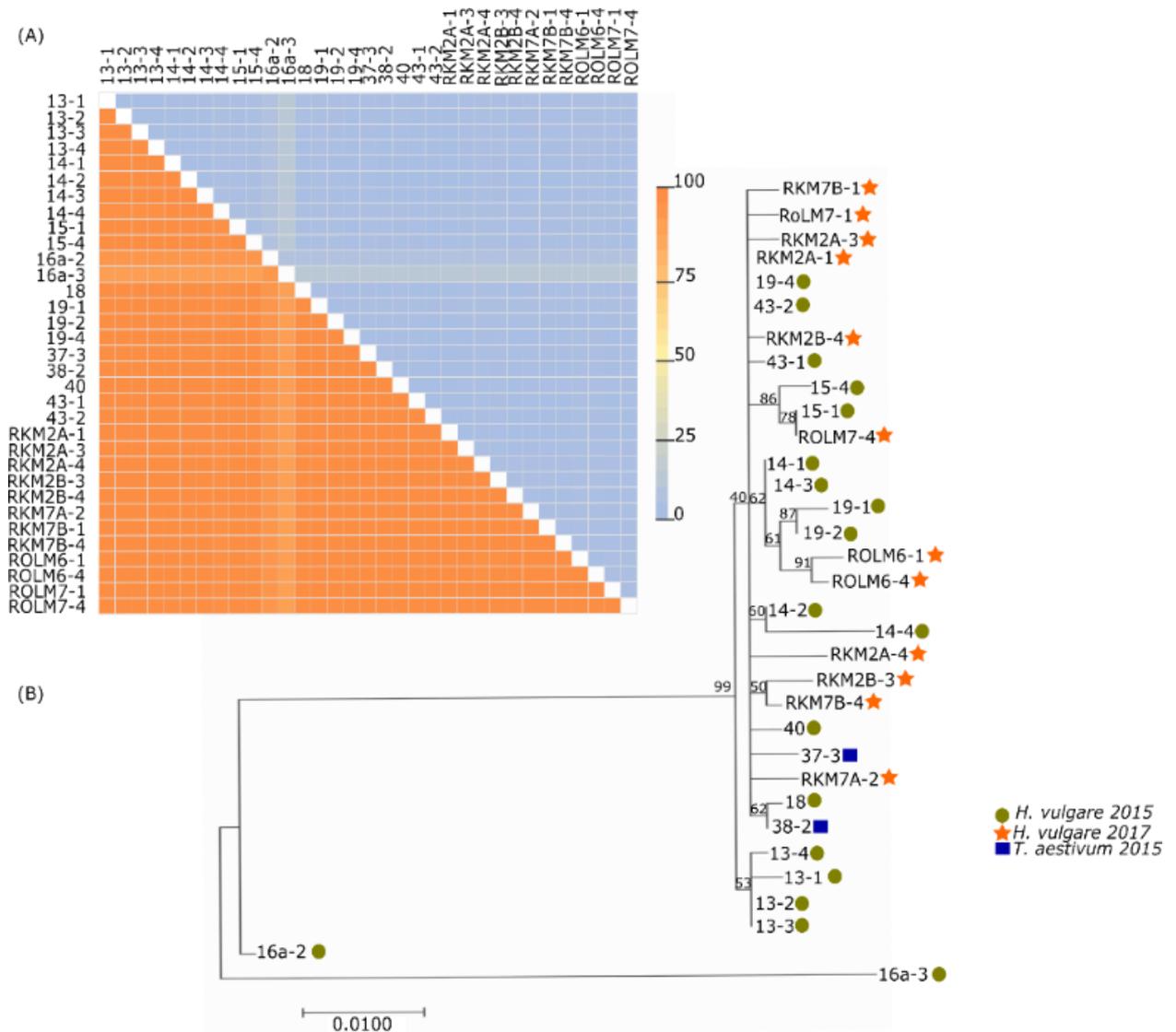


Figure 5. (a) Nucleotide identities (%) of Swedish BYDV isolates from 2015 and 2017 disease outbreaks. Upper triangle represents % difference and lower triangle % identity (b) Maximum likelihood (ML) phylogeny based on 789 nucleotides of the CP gene of BYDV isolates from wheat and barley in the 2015 and 2017 disease outbreaks. Values above nodes indicate bootstrap values. Ts/Tv: 2.02, SBL: 0.17 and Log likelihood: -1883.46. Scale bar indicates nucleotide substitutions per site.

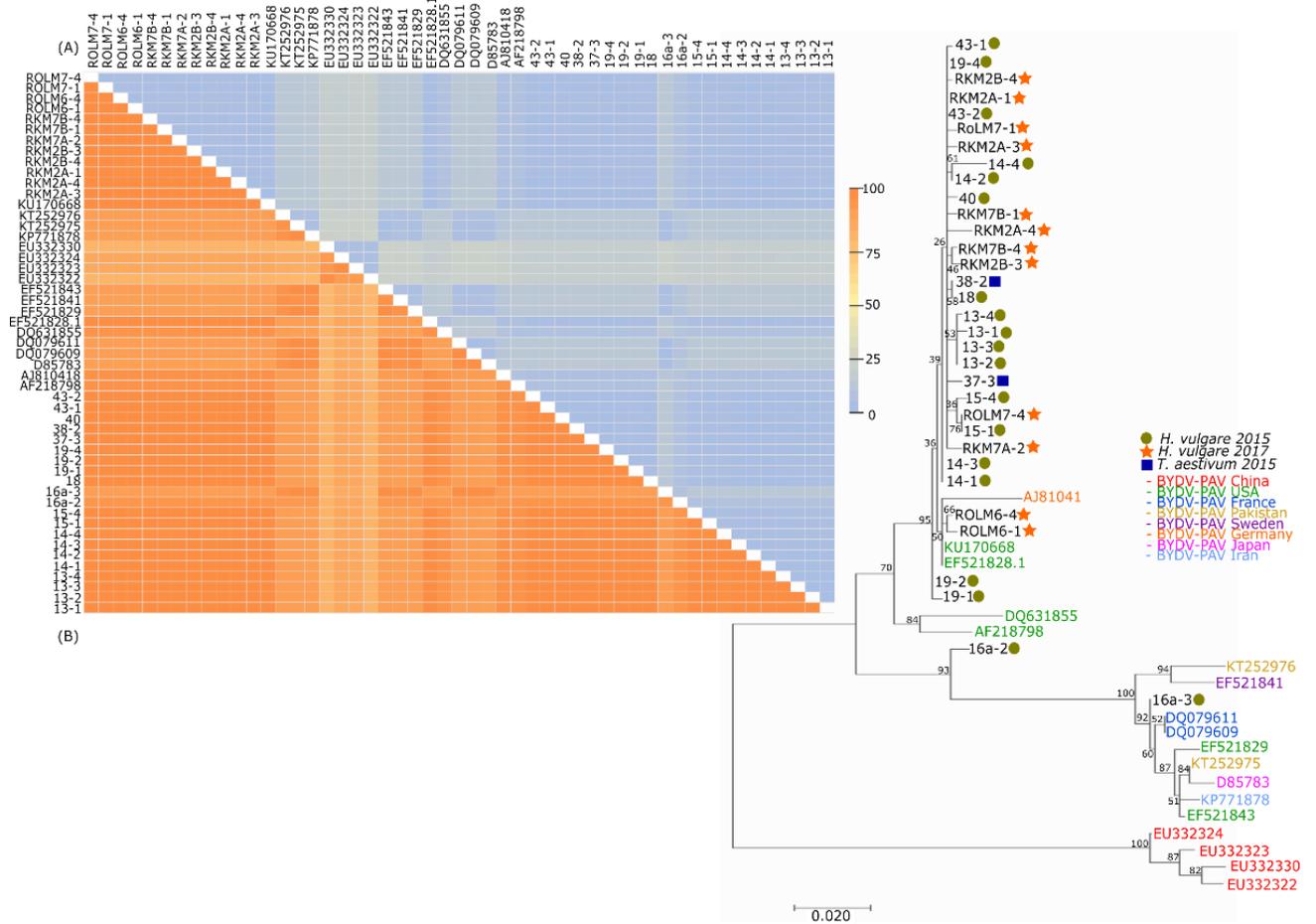
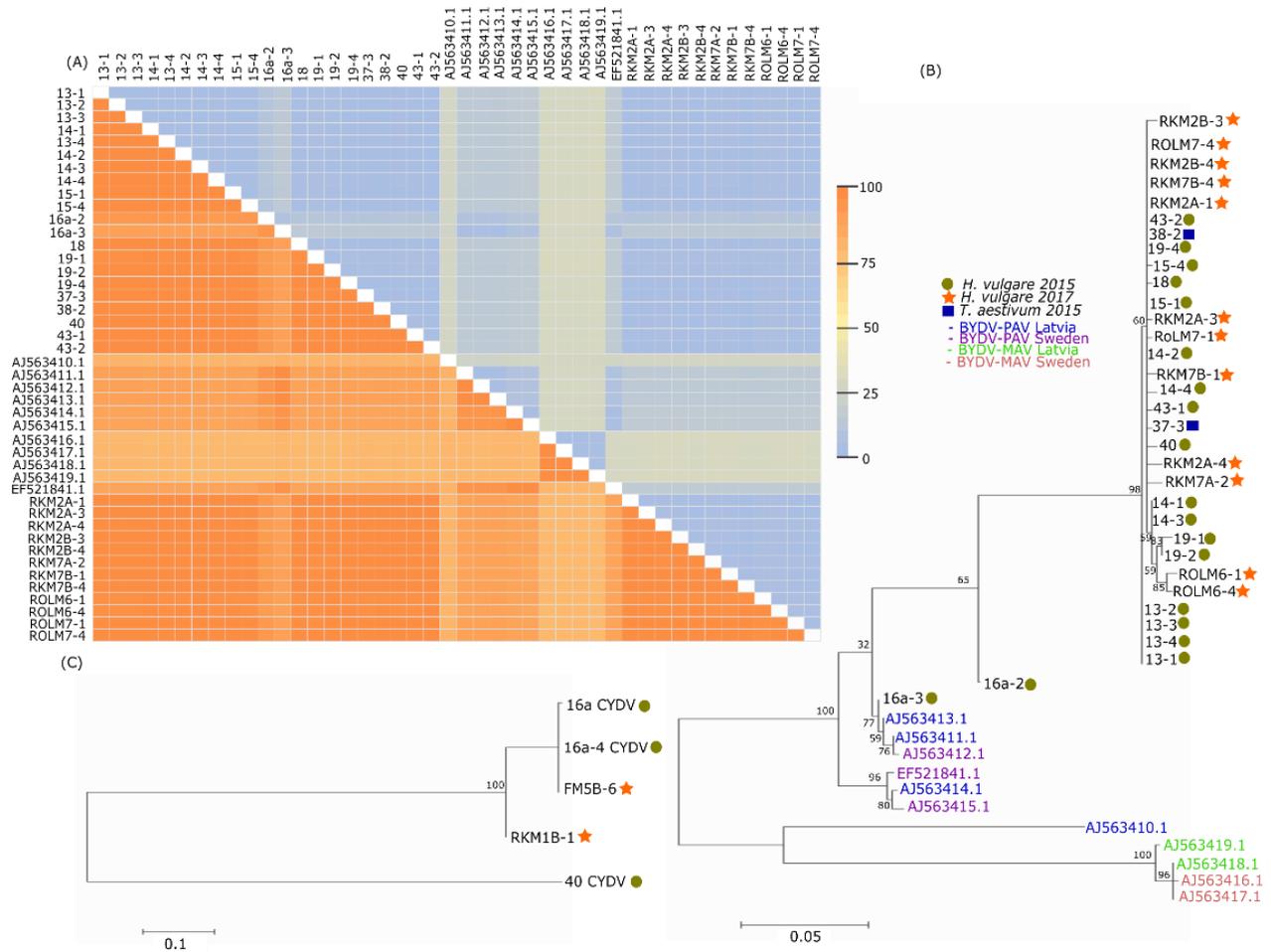


Figure 6. Swedish BYDV isolates from wheat and barley form a distinct group separate from global BYDV-PAV isolates and may represent BYDV-PAS. (a) Nucleotide identity (%) of Swedish BYDV isolates from 2015/17 and global BYDV-PAV isolates. Upper triangle represents % difference and lower triangle % identity (b) Maximum likelihood (ML) phylogeny based on 789 nucleotides of the CP gene of BYDV isolates from wheat and barley in the 2015 and 2017 disease outbreaks, and global BYDV isolates. Values above nodes indicate bootstrap values. Ts/Tv: 1.85, SBL: 0.47 and Log likelihood: -3126.38. Scale bar indicates nucleotide substitutions per site.



**Figure 7.** (a) Nucleotide identity (%) of Swedish BYDV isolates from 2015/17 and Swedish and Latvian isolates from a previous study. Upper triangle represents % difference and lower triangle % identity (b) Maximum likelihood (ML) phylogeny based on 502 nucleotides of the CP gene of BYDV isolates from wheat and barley in the 2015 and 2017 disease outbreaks, and Swedish and Latvian isolates from a previous study. Values above nodes indicate bootstrap values. Ts/Tv: 1.34, SBL: 0.59 and Log likelihood: -2042.18. (c) ML phylogeny of Swedish BYDV isolates from 2015/17 having shorter nucleotide lengths, 242 nt – 429 nt. Values above nodes indicate bootstrap values. Ts/Tv: 2.09, SBL: 1.36 and Log likelihood: -731.93. Scale bar indicates nucleotide substitutions per site.

## 4 Discussion

The current study used the diagnostic test of DAS-ELISA, for detecting the presence of BYDV-PAV, BYDV-MAV and CYDV-RPV infection of winter wheat, winter barley and winter rye during disease outbreaks in 2015 and 2017. The samples were further tested using RT-PCR, sequencing and phylogenetic analysis. The use of these tools in combination formed part of an initial serological identification with DAS-ELISA and more refined approach using sequence-specific primers for BYDV species and maximum likelihood phylogenetic analysis, to determine firstly, the diversity of isolates present during the disease outbreaks, and secondly to investigate the potential presence of transcapsidation or phenotypic mixing during disease outbreaks.

DAS-ELISA analysis identified BYDV-PAV, BYDV-MAV and CYDV-RPV as present in winter barley and winter wheat during the 2015 disease outbreak, the same was true of winter barley during the 2017 disease outbreak, while winter rye was only positive for BYDV-PAV and BYDV-MAV. When the samples were further tested by RT-PCR and sequence analysis the identified viral species were BYDV-PAV, BYDV-PAS and BVG. No BYDV-MAV or CYDV-RPV was identified from sequence analysis. The most closely related BYDV-PAV isolates were from USA and Germany, whilst for BYDV-PAS isolates from grass species in New Zealand were most similar. Previous assessments of BYDV diversity in Sweden have shown that BYDV is present in both wild and cultivated grass species, in which the former act as sources of inoculum (Eweida and Rydén, 1984). It has been demonstrated that wild grasses may be free of disease symptoms whilst cultivated species are not (Bisnieks et al., 2004). Visual evidence from the disease outbreak showed areas of cultivated land with leaf yellowing and stunting, thus initiating the project, however, no visual symptom score (VSS) was determined in field conditions. Phylogenetic analysis showed that most BYDV isolates collected in disease outbreaks in 2015 and 2017 were closely related, with nucleotide identities >99%, whilst isolates 16a-2 and 16a-3 had lower nucleotide identities. There was no identifiable difference between isolates collected from different plant species or at different sampling times. Within, a broader context, isolates from the disease outbreaks of 2015 and 2017 grouped with isolates from Germany and USA representing a distinct BYDV-PAS group, isolate 16a-3 representing a true BYDV-PAV type grouped with BYDV-PAV isolates from Sweden and France and isolate 16a-2 representing an intermediary between BYDV-PAV and BYDV-PAS was separate from both groups. The well-supported BYDV-PAS isolate grouping was separate from previously identified Swedish and Latvian BYDV-PAV isolates. A previous study on diversity of BYDV in Sweden (Bisnieks et al., 2004) did not indicate the presence of BYDV-PAS, however, the findings from this study are in agreement with the previous study of identifying BYDV-PAV as a causal agent during the BYD disease outbreaks in Sweden (Bisnieks et al., 2004). Later on, also BYDV-PAS has been identified during B/CYDV infection in cereals and wild grasses of Sweden, indicating that BYDV-PAS is part of the B/CYDV infection cycle (Kvarnheden, 2011). During the BYD disease outbreak of 2015 in Sweden, BYD disease was affecting also Polish barley (Jeżewska et al., 2016), and BYDV-MAV and BYDV-PAV were then detected using DAS-ELISA with high occurrence throughout the country (Jeżewska et al., 2016). Luteoviruses and BYDV have been extensively studied and a study on BYDV diversity and evolution identified 22 recombination events, including recombinants derived from parental recombinant lines in specific geographic locations (Boulila, 2011; Wu et al., 2011). These recombination events have allowed for the emergence of varying genotypes of BYDV globally. Most notable in this assessment is the identification of the sites of recombination, located in the genes encoding RdRp and CP (Wu et al., 2011). Luteovirid studies have identified regions of diversity among genomes, with the RdRp region located at ORF1 and ORF2, 3' region of the CP gene, the overlapping region of ORF2 and the CP gene and 5' end of the CP gene as sites of higher diversity (Pagán and Holmes, 2010). In particular it should be noted that these regions correspond to the differences resulting in a proposed alternative classification of

BYDV-PAV (BYDV-PAV-I), BYDV-PAS (BYDV-PAV-II) and BYDV-PAV-III (Wu et al., 2011), not established by the International Committee on the Taxonomy of Viruses (ICTV). BYDV-PAS was first recorded in the USA (Chay et al., 1996), as isolate BYDV-PAV 129 and later recognized as a separate virus species (Mayo, 2002). It was noted that the distinguishing characteristic of BYDV-PAS from that of BYDV-PAV is a ~14% difference in the CP sequence region (Chay et al., 1996). A critical factor in the designation of what constitutes viral species has been the adoption of the rule that amino acid sequence differences of greater than 10% in gene products, may be considered to distinguish novel species (Domier, 2011). The present project utilised the CP region of the BYDV and CYDV genome to identify virus species, as previously noted the CP region along with RdRp and RTD are variable sites at which recombination has taken place (Ali et al., 2017; Pagán and Holmes, 2010; Wu et al., 2011). It highlights that the use of a single genomic region for BYDV identification is inadequate, as the inherent complexity of the genome is omitted and may result in misidentification of isolates, most especially for BYDV-PAV and BYDV-PAS. Whilst in the current project isolates were closely grouped with one another and GenBank BYDV-PAS isolates, it does not provide information on how the BYDV-PAS and BYDV-PAV isolates from the 2015 and 2017 disease outbreaks are related to global isolates beyond the CP region. Whole genome sequencing (WGS) represents a complete assessment strategy, as the focus is shifted from selected genomic regions and variability within and between species may be more clearly defined. A recent study has shown that when using WGS nucleotide identity at 71% for species demarcation, BYDV-PAV, BYDV-MAV and BYDV-PAS are grouped as isolates of a single virus taxon *Barley yellow dwarf virus*, and BYDV-kerII and BYDV-kerIII form a separate species, *Barley yellow dwarf virus Kerguelen*, all of which are comprised of multiple BYDV strains (Ali et al., 2017). However, the use of a 71% nucleotide identity designation does require further study as using this methodology results in alternate taxonomic groupings. Future studies should focus on WGS techniques to improve understanding variability at other genomic regions, most especially for CP, RTD and RdRp and the relationship between this variability and disease symptom development.

BYDV-PAS has been identified globally in different countries and varying environmental and agricultural contexts such as the Czech Republic (Jarošová et al., 2013), Poland (Trzmiel, 2017), Alaska USA (Robertson and French, 2007), Iran (Pakdel et al., 2010), Pakistan (Siddiqui et al., 2012), New Zealand (Delmiglio, 2008) and France (Mastari et al., 1998). A more recent study on BYDV-PAS infection in the Czech Republic showed that BYDV-PAS was the most widespread BYDV, found in both wild grasses and cultivated cereals such as winter and spring barley, wheat, oat and maize (Jarošová et al., 2013). The widespread infections of BYDV-PAS thus formed the basis for an investigation of BYDV-PAS vector species, with *S. avenae* and *R. padi* identified as common during the field study period, in which aphid species tested positive overwhelmingly for BYDV-PAS, but were also positive for BYDV-PAV (Jarošová et al., 2013). The results show that these BYDVs share vector species and that mixed infections of BYDV-PAV and BYDV-PAS are possible within host plants (Jarošová et al., 2013). Presently in Sweden, information on aphid species, their estimated population size and migration is collected at several locations throughout the country to assist for disease outbreak predictions (Sigvald, 2012). The long-term data on aphid migration along with diagnostic analysis of infected plant material may be used to determine the prevalence of BYDV-PAS, BYDV-MAV, BYDV-PAV and CYDV-RPV during disease outbreaks and provide further insights on BYDV-PAS vector species and BYD disease epidemiology.

In a mixed infection of BYDV-PAV and BYDV-PAS, BYDV-PAV had a higher viral titre compared to BYDV-PAS, and a single infection with BYDV-PAV in susceptible plants resulted in greater visual symptoms than a single infection with BYDV-PAS (Jarošová et al., 2013). There was no visual symptom scoring within the current project and thus the severity of infection cannot be evaluated alongside diagnostic data. To this point, in a mixed infection of wheat distinct differences in the viral titres of BYDV-PAV and BYDV-PAS have been demonstrated (Hall and Little, 2013). During mixed infections BYDV-PAV had a higher viral load compared to BYDV-PAS, irrespective of the time period between subsequent infection and which virus formed the basis of initial infection (Hall and Little, 2013). However, BYDV-PAS was demonstrated to confer a relative cross-protection during a shorter inoculation window between initial and secondary infection when BYDV-PAS was used in the initial infection (Hall and Little, 2013). Early experiments on cross-protection for BYDV have focused on BYDV-PAV and BYDV-MAV (Wen et al., 1991), however more recent studies have not been conducted on these viruses or other luteovirid species including BYDV-PAS and CYDV-RPV. Understanding the relationship of mixed infections of BYDV-PAS and BYDV-PAV would assist in better understanding BYD epidemiology, providing insight to questions such as; If BYDV-PAS forms part of the initial infection does this confer cross-protection to BYDV-PAV?, identifying the threshold of BYDV-PAS titre in the host at which cross-protection is initiated, the plant growth stage of cross-protection and information on symptom development during mixed infections.

In agreement with the findings of previous studies (Hall and Little, 2013, Jarošová *et al.*, 2013), there is the inability by DAS-ELISA to distinguish between BYDV-PAV and BYDV-PAS, with many samples testing positive for BYDV-PAV. This makes it difficult to distinguish cases of single infections with BYDV-PAV or BYDV-PAS or double infections and further highlights the need to develop specific antibodies for BYDV-PAS and BYDV-PAV to facilitate rapid detection of virus infection which is then supplemented with PCR techniques using species-specific primers. DAS-ELISA was used to determine if transcapsidation or phenotypic mixing occurred between BYDV-MAV and BYDV-PAV, it has not been possible to distinguish the virions of these viral species. Recent studies by electron microscopy have been undertaken to examine the structural nature of luteovirid capsids, particularly on how they may differ between species. It has been shown by computational analysis that the capsid is composed of a S-domain largely comprising the outer shell of the virion and an R-domain, identified to interact with viral RNA as well as a number of cross-links between sites (Alexander *et al.*, 2017). Most importantly these studies have been conducted on *Turnip yellows virus* and *Potato leaf roll virus*, not on BYDV as the isolation of virions for mass spectrometry (MS) study still requires optimization. To better examine the evidence of transcapsidation or phenotypic mixing would require understanding how viral RNA interacts with the capsid shell, critically identifying the sites of RNA-capsid interaction and if these interactions are characterized by a high specificity as for aphid-virus interactions. Additionally the viral RNA release protocol (Harju *et al.*, 2005) should be optimized for B/CYDVs to create a single pipeline for DAS-ELISA followed by PCR.

In the current study an isolate of BVG was identified from barley during the BYD disease outbreak in 2015. BVG was likely detected by CYDV-RPV in DAS-ELISA and by CYDV-RPV primers during RT-PCR. BVG was first identified in South Korea from cultivated barley (Zhao *et al.*, 2016). Since then, BVG has been identified in the USA in *P. virgatum* imported from the Netherlands (Kumar *et al.*, 2018), as well as in South Korea in *P. miliaceum* (Park *et al.*, 2017a) and *S. italica* (Oh *et al.*, 2017). The genomic organization of BVG is consistent with that of viruses within the genus *Polerovirus* (Zhao *et al.*, 2016). The identified Korean isolates have all been collected from cultivated plant material in which infected plants had symptoms typical of BYDV infection, with additional symptomology on leaves of yellow streaking and green mosaic (Oh *et al.*, 2017; Park *et al.*, 2017a; Park *et al.*, 2017b; Zhao *et al.*, 2016). The prevalence of BVG has been evaluated in *S. italica*, of 133 symptomatic plants 39 tested positive (29%) (Oh *et al.*, 2017), whilst in *P. miliaceum* 17 of 81 plants were positive (21%) (Park *et al.*, 2017). These studies used NGS techniques to identify BVG, highlighting the need for NGS evaluation to identify novel species as well as to gain improved understanding of the viruses causing BYD disease. The presence of BVG in Sweden indicates the widespread nature of BYDVs globally and the rapid spread of these viruses. In particular, aphid vector data should switch from a focus on abundance data but also include PCR analysis. The persistent transmission system via aphids could also mean that BVG is maintained in wild grasses at low abundance till favourable conditions arise. A secondary focus should be on polar jet transport of aphids to predict future and new emerging viruses from far afield geographic locations. BVG diversity has not been evaluated to date, and as previously discussed BYDVs are highly variable and have undergone several recombination events, thus new variants of BVG and BYDV-PAV, BYDV-MAV are likely to occur.

The results from this study are encouraging in terms of identifying the common BYDVs present in Sweden and increase the understanding of these virus which cause BYD disease in the region. Future studies should use NGS techniques to underpin studies on B/CYDVs, as there have been a number of identified recombination events, and therefore it is important to understand the type of differentiation which has taken place in southern Swedish isolates compared to global isolates. There was no scoring of symptoms in field, which would assist in determining the severity of disease in field and identification of strains which are important agriculturally. Future work in this area should focus on visual scoring along with information on the cultivars planted, extended to include a broader geographic area, or increased intensity of collection for a specific region.

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