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POPULAR SCIENCE:

Barley yellow dwarf (BYD) disease is affecting a wide range of plants in the family Poaceae, such as oat, wheat, barley, rice and fodder crops worldwide. This causes a significant yield reduction every year. Causal agents of the disease are BYD-associated viruses, which belong to family *Luteoviridae* and are transmitted by aphids. The family *Luteoviridae* contains several species and a recent taxonomic classification recognized 36 species. The taxonomy within the family is based on the arrangement of the genome assessed by DNA sequencing. The genome arrangement of an organism provides a background to understand its genetics and biology. Based on the genome arrangement, a new virus variant was discovered in Latvia by comparing nucleotides from the coat protein (CP) – encoding region of Latvian and Swedish isolates. The new variant is BYDV-PAV-Sal1, proposed to belong to the new species Barley yellow dwarf virus-oat yellowing virus (BYDV-OYV). The isolate shared closest relationship with a new Chinese isolate (BYDV-PAV-CN). Isolates of BYDV-OYV have also been found recently in grasses in Sweden. Thus, this study was designed to reveal the relationship between BYDV-OYV and BYDV-PAV and to characterize a Swedish isolate of BYDV-OYV based on its partial genome sequence. The partial genome sequence of BYDV-OYV was obtained in three different fragments using molecular techniques. A partial genome assembly of the three fragments was aligned and compared with BYDV-PAV-Sal1, BYDV-PAV-CN and 15 other isolates of the family *Luteoviridae*. The results revealed that the Swedish BYDV-OYV isolate and BYDV-PAV-Sal1 showed a close relationship. BYDV-OYV and BYDV-PAV-CN were found to be distinct species based on the recent agreement that viruses with a variation at the amino acid level exceeding 10% for any viral gene product could be classified as belonging to separate species within the family *Luteoviridae.*

Abstract

Barley yellow dwarf (BYD)-associated viruses are economically important in a wide range of cereals and grasses worldwide. These viruses belong to family *Luteoviridae* and are efficiently transmitted by aphids in a circulative and non-propagative manner. BYD-associated viruses have a genome of single-stranded positive sense RNA (+ssRNA). Even if isolates of BYD-associated viruses from different parts of the world show significant divergence in pathogenicity and genetic structure, the evolutionary process and the genomic structure of new viral species are poorly known. In this study, BYDV-OYV, which has been proposed to be a distinct tentative virus species and infects oat (*Avena sativa*), Triticale and grasses in Sweden and Latvia, was investigated to characterize its genomic structure and relationship with other members of family *Luteoviridae*. To address these issues, a partial region of the BYDV-OYV genome was determined for one Swedish isolate. The sequence consisted of 2,792 nucleotides and contained 4 open reading frames and 3 un-translated regions. The genomic structure of BYDV-OYV resembled those of other species in family *Luteoviridae*. Sequence comparisons including 15 isolates of the genera *Luteovirus* and *Polerovirus*, showed that BYDV-OYV shared highest nucleotide identity with BYDV-PAV-CN (AY855920), BYDV-PAV isolate 05ZZ4 (EU332321), BYDV-PAV isolate 05ZZ13 (EU332327) and BYDV-PAV isolate 05GG5 (EU332310) at 82.8%, 82.6%, 82.6% and 82.5%, respectively. A phylogenetic analysis confirmed that BYDV-OYV is related to other viruses in the genus *Luteovirus*, but that it is separate from the other known luteoviruses. The deduced amino acid sequence identity of ORF3 (coat protein) between BYDV-OYV and BYDV-PAV-CN was only 87%. Thus, BYDV-OYV should be regarded as a species distinct from BYDV-PAV-CN.

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1. General introduction

Barley yellow dwarf (BYD)-associated viruses constitute the most economically important group of viruses infecting plants of the family Poaceae, including oat, wheat, barley and rice, as well as pasture and wild grasses all over the world (Power & Remold, 1996; D'Arcy, 1995; Irwin & Thresh, 1990). Barley yellow dwarf virus (BYDV) was first identified in California, USA as a new virus in cereals (Oswald & Houston, 1951). BYD-associated viruses belong to family *Luteoviridae* (Domier, 2012) and have a genome consisting of single-stranded positive-sense RNA. BYD-associated viruses are transmitted only by aphids in a circulative and non-propagative manner (Hogenhout et al., 2008; Gray & Gildow, 2003). Economic losses due to these viruses around the world are difficult to estimate because of insufficient molecular information and symptoms that resemble the effects of other biotic and abiotic factors. At the same time the evolutionary potential of BYD-associated viruses is very high like other RNA viruses that cause diseases on humans, animals and plants (Elena & Sanjuan, 2008). Initially, five different strains of BYDV were distinguished by their aphid vector specificities (Rochow & Muller, 1971; Rochow, 1969). These strains were later classified as separate species, and the number of recognized species within the family *Luteoviridae* has now increased to 34 (Domier, 2012). The increasing number of new virus species in family *Luteoviridae* creates a fascinating area of studies regarding their diversification pattern and taxonomic classification using sequence analyses. The classification is also important for investigation of virus epidemics and development of appropriate management strategies.

1.1. Family *Luteoviridae*

The family *Luteoviridae* comprises the three genera *Luteovirus*, *Polerovirus* and *Enamovirus* which are distinguished by their biological properties, serological properties and genome organization. Among them, 23 species are classified into the three genera: *Luteovirus* has 7 species, *Polerovirus* has 15 species and *Enamovirus* comprises only one species. The remaining members have not yet been classified and remain unassigned.

1.2. Genome structure and function of family *Luteoviridae*

The genome structure and nucleotide sequences of a large number of virus genomes, including those of viruses in the family *Luteoviridae*, have been determined after a rapid development of molecular techniques. The three genera of family *Luteoviridae* are clearly distinguished by differences in the genome structure and nucleotide sequence (Fig. 1).

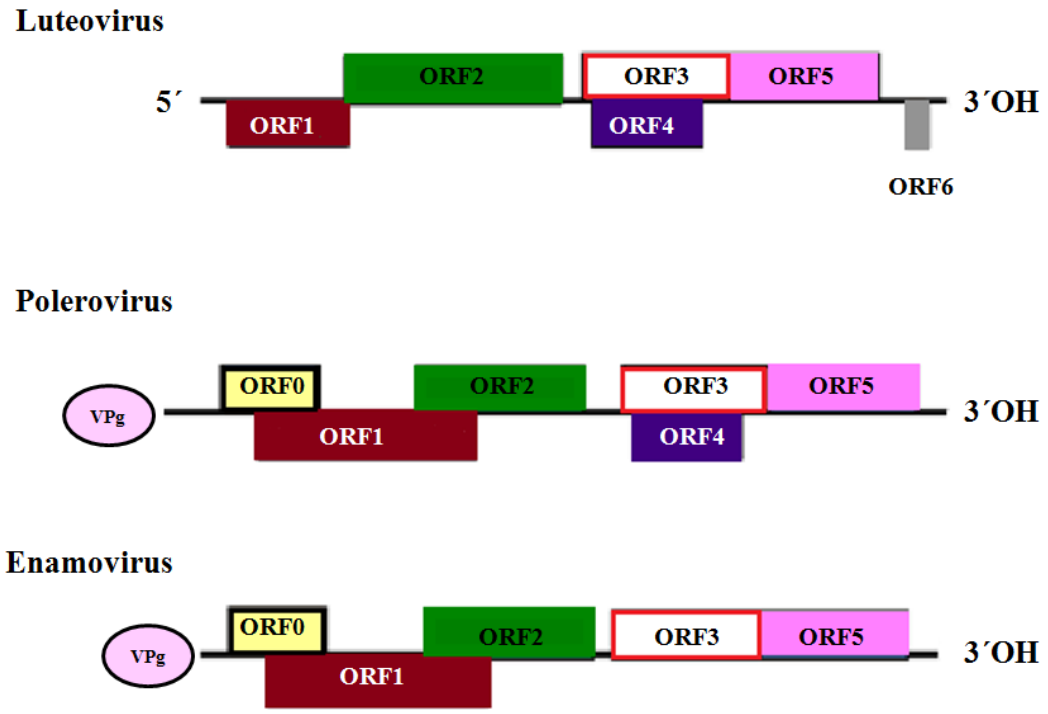

Fig. 1. Genome organization of genera *Luteovirus*, *Polerovirus* and *Enamovirus* within the family *Luteoviridae*.

The genomes of the three genera in family *Luteoviridae* harbour five or six open reading frames (ORFs), which have been predicted to encode proteins of between 4 kDa and 132 kDa (Fig. 1) (Domier, 2012). The 5´ part of the genome of both genera *Polerovirus* and *Enamovirus* contains ORF0, which encodes a suppressor of RNA silencing (Mangwende et al., 2009), but that is absent from viruses of the genus *Luteovirus*. ORF0 is also responsible for symptom induction and determination of viral host range, and it overlaps with ORF1. ORF1 and ORF2 overlap and encode the replication-related proteins. The 3´ half is mostly conserved among all members of *Luteoviridae*. ORFs 3, 4 and 5 are highly similar in arrangement for *Luteovirus* and *Polerovirus* (Domier, 2012), but ORF4 is absent from *Enamovirus*. ORF4 has been shown to be necessary for long distance movement of some

luteoviruses and poleroviruses (Chay et al., 1996). ORF3 encodes the coat protein (CP). ORF5 is necessary for aphid transmission (Brault et al., 2005; Gray & Gildow, 2003; Chay et al., 1996) and it may also be involved in systemic virus movement within plants (Peter et al., 2009; Ziegler-Graff et al., 1996) and phloem limitation (Peter et al., 2009). The genome of viruses in the genus *Luteovirus* contains ORF6, which is situated at the 3´ end of the genome, but its function has not yet been elucidated (Domier, 2012).

1.3. Economic importance of family *Luteoviridae* and BYD-associated viruses

Twenty six members were assigned into family *Luteoviridae*in 2005 (Fauquet et al., 2005), but this number has now increased (Table 1) (Domier, 2012) because of further studies, the availability of advanced detection methods and virus evolution. Many members of family *Luteoviridae* are regarded as serious pathogens in global agriculture. *Potato leaf roll virus* (PLRV), which belongs to the genus *Polerovirus,* may cause yield losses in potato cultivation from 10% in the most tolerant varieties to around 95% in the most susceptible varieties (Watson & Wilson, 1956). Over the last decades, many researches have been demonstrated that PLRV caused huge losses of potato tubers and reduced tuber size (Wang et al., 2011; Valkonen, 2007). Experimental data showed that PLRV infected seeds gave 60% less of total tuber yield and 88% less of market value than potato plants derived from healthy seed tubers (Hamm & Hane, 1999).

Luteoviruses also create mixed infection with related and unrelated viruses. These mixed infections are causing serious disease epidemics, for example “Beet yellowing viruses” are causing a disease on beet leaves upon co-infection of *Beet yellows virus* (BYV, family *Closteroviridae*, genus *Closterovirus*), *Beet western yellows virus* (BWYV, family *Luteoviridae,* genus *Polerovirus*) and *Beet chlorosis virus* (BChV, family *Luteoviridae,* genus *Polerovirus*) (Liu et al., 2001; Hauser et al., 2000). The mixed infections have probably played an important role in the evolution of luteoviruses and other viruses. Within family *Luteoviridae,* BYD-associated viruses have been considered to be the most economically important ones, since they are disease agents in many cereals serving as staple crops. The disease causes greatest yield losses from infection at early stages to stem extension (Thackray et al., 2005; Mckridy et al., 2002). For example, early sown wheat crops, from mid April to late May, that are exposed to early migration and infestation of aphids, face grain yield loss of

up to 60% in a Mediterranean-type environment (Thackray et al., 2009). In an another study, around 100 aphids per day per plant are estimated to cause around 1% grain yield loss on wheat in Brazil (Savaris et al., 2013). However, aphids also cause sporadic yield losses through direct feeding (Michael, 2002).

Many phytopathogenic infections, including those of luteoviruses, became more serious from the early 20th century when larger areas were used for monoculture. Monoculture constituted a great habitat for luteoviruses, like other pathogens, to evolve and spread. Thus, since the 20th century various control measures have been implemented to avoid crop losses (Robert & Lemaire, 1999). In the beginning, the crops were protected by applying insecticides (aphicides) to viral vectors. Although insecticide application might substantially increase the yield as well as being a cheap technique for controlling luteoviruses (Stoetzer et al., 2014), it also has caused increased negative effects on the environment and on human health (Orden et al., 2004; Pimentel et al., 1992; Babu & Hallam, 1989). Accordingly other control measures are needed to eliminate the negative impact caused by insecticides. One strategy is integrated pest management, which includes, e.g., combinations of altering sowing dates with insecticide applications for viral vectors (McGrath & Bale, 1990). However, it is not effective during all circumstances, since it mainly depends on vector population dynamics (Morgan, 2000), plant genotypes, virus species (Baltenberger et al., 1987), environmental conditions, monitoring tools and quarantine activities. Another control measure is the use of natural resistance genes, which have been identified in some major crops such as the *Yd*2 gene in barley (Schaller et al., 1964) and *Bdv*1 in wheat (Makkouk & Kumari, 2009). However, these genes fail to provide protection against all BYD-associated viruses. Tolerant lines havealso been identified against BYDV (Burnett et al., 1995), but the tolerance is multigenic and difficult to manipulate through conventional breeding. Researchers have tried to introduce artificial resistance genes to disrupt replication of BYDV (Miller & Young, 1995). However, as a result of rapid evolution, viruses may evade plant host immune systems (Holmes, 2009; Garcia & McDonald, 2003; McDonald & Linde, 2002). According to recent research, luteoviruses and poleroviruses may inhibit plant defense mechanisms, such as RNA silencing pathways (Burgyan & Havelda, 2011).

Table 1. Members in the family *Luteoviridae* (Domier, 2012).

	Species name	Names of isolates	Abbreviations	Sequence accession numbers
Genus *Luteovirus*				
1	*Barley yellow dwarf virus*-MAV	Barley yellow dwarf virus-MAV – PS1	BYDV-MAV-PS1	D01213
2	*Barley yellow dwarf virus-PAS*	Barley yellow dwarf virus-PAS – 129	BYDV-PAS - 129	AF218798
3	*Barley yellow dwarf virus-PAV*	Barley yellow dwarf virus-PAV – Australia	BYDV-PAV - AUS	X07653
4	*Bean leaf roll virus*	Bean leaf roll virus - Michigan	BLRV-MI	AF4451393
5	*Rose spring dwarf-associated virus - California*	Rose spring dwarf-associated virus - California	RSDaV-CA	EU024678
6	*Soybean dwarf virus*	Soybean dwarf virus – Tas-1	SbDV-TAS-1	L24049
Members of genus *Luteovirus* which have not been approved as species				
7	Barley yellow dwarf virus-GAV		BYDV-GAV	AY220739
Genus *Polerovirus*				
1	*Beet chlorosis virus*	Beet chlorosis virus – 2a	BChV-2a	AF352024
2	*Beet mild yellowing virus*	Beet mild yellowing virus – 2ITB	BMYV-2ITB	X83110
3	*Beet western yellows virus*	Beet western yellows virus– USA	BWYV-US	AF473561
4	*Carrot red leaf virus*	Carrot red leaf virus – UK1	CtLRV-UK1	AY695933
5	*Cereal yellow dwarf virus-RPS*	Cereal yellow dwarf virus-RPS – Mex1	CYDV-RPS-Mex1	AF235168
6	*Cereal yellow dwarf virus-RPV*	Cereal yellow dwarf virus-RPV – NY	CYDV-RPV - NY	L25299
7	*Chickpea chlorotic stunt virus*	Chickpea chlorotic stunt virus – Et-fb-am1	CpCSV-Et-fb-am1	AY956384
8	*Cucurbit aphid-borne yellows virus*	Cucurbit aphid-borne yellows virus– N	CABYV-N	X76931
9	*Melon aphid-borne yellows virus*	Melon aphid-borne yellows virus - Beijing	MABYV-BJ	EU000534
10	*Potato leaf roll virus*	Potato leaf roll virus – UK:Scotland	PLRV-UK	D00530
11	*Sugarcane yellow leaf virus*	Sugarcane yellow leaf virus– Florida	ScYLV-FL	AF157029

12	*Tobacco vein distorting virus*	Tobacco vein distorting virus – China:Longlin	TVDC-CN	EF529624
13	*Turnip yellows virus*	Turnip yellows virus FL-1	TuYV-FL1	X13063
Members of genus *Polerovirus* which have not been approved as species				
14	Cotton leafroll dwarf virus		CLRDV	GQ379224
15	Suakwa aphid-borne yellows virus		SABYV	FJ425878
Genus *Enamovirus*				
1	*Pea enation mosaic virus-1*	Pea enation mosaic virus-1– WSG	PEMV-1-WSG	L04573
Unassigned species in the family *Luteoviridae*				
1	*Barley yellow dwarf virus-GPV*	Barley yellow dwarf virus-GPV – 04FX6	BYDV-GPV-04FX6	EF174408
2		Wheat yellow dwarf virus-RPV	WYDV-RPV	FM865413
3	*Barley yellow dwarf virus-RMV*	Barley yellow dwarf virus-RMV - Illinois	BYDV-RMV-IL	Z14123
4	*Barley yellow dwarf virus-SGV*	Barley yellow dwarf virus-SGV – NY	BYDV-SGV-NY	AY541038
5	*Chickpea stunt disease associated virus*	Chickpea stunt disease associated virus - IC	CpSDaV-IC	Y11530
6	*Groundnut rosette assistor virus*	Groundnut rosette assistor virus – M16GCP	GRAV-M16GCP	AF195824
7	*Indonesian soybean dwarf virus*	Indonesian soybean dwarf virus– IND	ISDV-IND	
8	*Sweet potato leaf speckling virus*	Sweet potato leaf speckling virus– Peru	SPLSV-Peru	DQ655700
9	*Tobacco necrotic dwarf virus*	Tobacco necrotic dwarf virus– Japan	TNDV-JA	
Members of family *Luteoviridae* which have not been approved as species				
10	Chickpea yellows virus		CpYV	GQ118150
11	Lentil stunt virus		LSV	GQ118152

1.4. Disease symptoms of barley yellow dwarf

BYD-associated viruses are phloem-limited and interfere with translocation of photosynthesis products from cells in leaves to sieve tube elements through plasmodesmata (Gray, 1996;

Rochow & Duffus, 1981). Severe infection causes rolling, reddening or yellowing of leaves, stunted plants, inhibited root formation, delayed heading and reduced yield. However, disease symptoms may vary depending on the host plants and host plant genotypes. For instance, oats show reddening of the leaf blades along the vascular bundles, blasting of the florets, stunted growth and late heading (Yount et al., 1985), whereas infected barley and wheat show chlorosis or yellowing and stunted growth. Additionally, un-matured grains of infected plants are easily infected with other pathogens, especially fungal pathogens (D'Arcy, 1995). Until the discovery of BYDV (Oswald & Houston, 1951), it was considered that the symptoms were caused by environmental conditions such as temperature, soil moisture and soil fertility. Although these symptoms are caused by the virus, still it is very difficult to detect the virus and estimate yield losses using only symptomatology which may be masked by other factors.

1.5. Basic virological and biological characteristics of BYDV

BYD-associated viruses have a genome consisting of positive sense single-stranded RNA (+ssRNA) and the total genome size is around 5.6 to 6.0 kb. The virions are hexagonal particles with a diameter of 24-25 nm. They are not enveloped and genomic RNA is encapsidated by CP with a molecular weight of 21 to 23kDa (Domier, 2012).

BYDVs are completely dependent on their vectors for their transmission (Fiebig et al., 2004). Viruses of each species are transmitted by a particular range of aphids (Rochow & Muller, 1971; Rochow, 1969). BYDV-PAV is efficiently spread by both the oat bird-cherry aphid (*Rhopalosiphum padi*) and the English grain aphid (*Sitobion avenae*; formerly *Macrosiphum avenae*). BYDV-MAV is effectively spread by the English grain aphid. BYDV-SGV is transmitted by the green bug (*Schizaphis graminum*) and rarely by the English grain aphid. BYDV-RMV is spread by the corn leaf aphid (*Rhopalosiphum maidis*) and also rarely by the oat bird-cherry aphid, the English grain aphid and the green bug. CYDV-RPV is transmitted by the oat bird-cherry aphid and rarely by the corn leaf aphid, the English grain aphid and green bug (Rochow, 1969). The virus names were mostly based on their vector specificity. Initially, vector specificity was a significant biological characteristic of the virus to distinguish the different virus serotypes.

1.6. Occurrence and impact of B/CYDVs in Sweden

In Sweden B/CYDVs were detected for the first time in cereal crops in 1957 (Lindsten, 1964) and the viruses can have a great economic impact on spring-sown cereals, especially on oats (Lindsten, 1977). The various virus isolates may differ in symptom induction on infected plants and transmissibility by their vectors (Lindsten, 1978). The vectors were mainly *R. padi*, *S. avenae* and *Metopolophium dirhodum*, which transmitted BYDV-PAV, BYDV-MAV and CYDV-RPV, respectively (Eweida, 1985). However, BYDV-PAV and BYDV-MAV were most frequently occurring in Sweden (Eweida & Oxelfelt, 1985; Eweida, 1986). The viruses overwinter in infected wild grasses, which act as a reservoir (Plumb, 1983). *Festuca pratensis* (Ängssvingel) and *Lolium perenne* (Engelskt rajgräs) are commonly infected grasses, which are susceptible to B/CYDVs (Lindsten & Gerhardson, 1969). Bird-cherry trees (*Prunus padus*) are used by *R. padi* for overwintering and then they migrate to cereals in the spring (Östman et al., 2003). BYDV-PAV, BYDV-MAV and CYDV-RPV were detected in spring sown cereals and grasses in a field survey that was conducted in 2000 and 2001 in Latvia (Bisnieks et al., 2002). Yield losses caused by *R. padi* have been estimated to reach up to 600 kg/ha or around 15% of yield loss in barley (Hallqvist, 1991). However the yield loss may vary based on the host plants' growth stage. For instance, the infection of a Swedish BYDV-PAV isolate on early growth stages of oats decreased yield of grain biomass and plant height (Bisnieks et al., 2005).

1.7. Taxonomy of BYD-associated viruses

1.7.1. Taxonomy of BYD-associated virus based on vector transmission

The taxonomy of BYD-associated viruses has gone through several phases. In the beginning, the taxonomy was based on biological characterizations, such as vector specificity, host range and symptomatology. Mainly, biological properties such as virus-vector relationships were used for biological classification of BYD-associated viruses (Rochow & Muller, 1971; Rochow, 1969). According to this classification, BYDV / CYDV isolates from New York were classified into five serotypes or strains: BYDV-MAV, BYDV-SGV, BYDV-RMV, BYDV-PAV and CYDV-RPV. After that, two more strains were added: BYDV-GPV, which is efficiently transmitted by *S. graminum* and *R. padi* (Wang et al., 1998; Cheng et al., 1996; Zhou et al., 1987), and BYDV-GAV, which is specifically transmitted by *S. graminum* and *S.*

avenae (Wang et al., 2001) in China. However, the vector based identification failed to classify all virus strains, because vector based characterization may not reflect the entire vector range of a particular virus strain. For instance, *S. graminum* is an efficient vector of some isolates of BYDV-PAV and CYDV-RPV, and *M. dirhodum* can be an efficient vector of some isolates of BYDV-MAV and BYDV-PAV (Gray et al., 1998). At the same time, this technique needs prior knowledge of the biology of the virus and biotype of the vector (Bencharki et al., 2000), access to one or several colonies of virus-free aphids, space for growth of test plants to inoculate the aphids and it also takes long time. Currently, to eliminate these constraints, several advanced methods are widely used for classification of virus isolates.

1.7.2. Taxonomy of BYD-associated virus based on serological properties

The serological properties of BYD-associated viruses were used to re-classify these viruses as species and identify their relationship with other members of family *Luteoviridae*. Serological methods, such as enzyme-linked immunosorbent assay (ELISA), tissue-blot immunoassay (TBIA), and serologically specific electron microscopy (SSEM), provide rapid and more specific information. Different ELISA techniques include double antibody sandwich (DAS-ELISA) or triple antibody sandwich (TAS-ELISA), and they are widely used for the identification of B/CYDVs (Lister & Rochow, 1978). Strain-specific antibodies have been established for six of the B/CYDVs: BYDV-MAV, BYDV-RMV, BYDV-SGV, BYDV-PAV, BYDV-GAV and CYDV-RPV (Wang et al, 2001; Lister & Rochow, 1979). It has also been possible to determine the serological relationships between different luteoviruses with ELISA tests. B/CYDVs were divided into two groups based on serological relationships. Group 1 contained MAV, PAV and SGV and group 2 included RPV and RMV (Martin & D'Arcy, 1990). Although DAS-ELISA is very effective in detection of luteoviruses in both infected plants and their vectors, specific ELISA tests are not available for all species and especially not for new unassigned species (Malmstrom & Shu, 2004). ELISA tests may also fail to detect viruses if the viral titer is low (Peter et al., 2009). Other serological techniques, such as TBIA, immunogold-labelling techniques and SSEM are rarely used for detection and classification purposes (D´Arcy et al., 1999).

1.7.3. Taxonomy of BYD-associated virus based on nucleic acid-based diagnostics

Nucleic acid-based diagnostics is being a very rapid and streamlined approach in modern classification and it also offers several advantages compared to the ELISA tests (Martin et al., 2000; Henson & French, 1993). Detection of phytopathogens has been revolutionized by this technique (Vincelli & Tisserat., 2008; James et al., 2006; Elnifro et al., 2000). Among them, B/CYDV could be easily detected and classified by nucleic acid-based techniques, especially reverse-transcription polymerase chain reaction (RT-PCR) which could detect B/CYDV in low-titre host plants or with small amount of samples (Nassuth et al., 2000; Figueira et al., 1997). The success of the RT-PCR process depends on suitable primers for the amplification of luteovirus sequences (Deb & Anderson, 2008; Vigano & Stevens, 2007; Malmstrom & Shu, 2004) or other +ssRNA viruses that are causing diseases on the same hosts as luteoviruses (Viswanathan et al., 2009) in both monoplex and multiplex RT-PCR. Furthermore, these viruses could be detected through RT-PCR in their aphid vectors (He et al., 2006; Liu et al., 2006).

In recent years, the complete or partial nucleotide sequences and genome organization of several BYD-associated virus isolates have been determined (Zhang et al., 2009). This has led to a major reorganization of the taxonomy. The viruses causing barley yellow disease are classified into two genera: BYDV-PAV, -MAV and -PAS within the genus *Luteovirus*; CYDV-RPV and -RPS within the genus *Polerovirus*; while BYDV-SGV, -GPV, and -RMV have not yet been allocated to any genus (Domier, 2012). Virus isolates identified as BYDV-PAV have recently been suggested to be reclassified into three distinct species based on nucleotide sequences, symptom induction in different hosts and antibody reaction: BYDV-PAV as PAV-I; BYDV-PAS as PAV-II and Chinese isolates of BYDV-PAV-CN as PAV-III (Liu et al., 2007). These species were separated based on the recent agreement that variation at the amino acid level exceeding 10% for any viral gene product could be applied as the single principle for separating species within the family *Luteoviridae* (Domier, 2012).

1.8. B/CYDVs detection based on CP gene and its breakthrough

Partial CP gene analysis of virus isolates positive in ELISA tests for BYDV-PAV and BYDV-MAV in Latvia and Sweden have been used to study genetic diversity (Bisnieks et al., 2004). In this study, a new variant, BYDV-PAV-Sal1, was discovered in Latvia. Although initially

the new variant was classified as BYDV-PAV based on detection by a PAV-specific antibody, it showed genetically clear variation from other BYDV species. The new variant PAV-Sal1 shared no more than 86% amino acid identity with other BYDV isolates. So, PAV-Sal1 was proposed to be a member of a new tentative species, Barley yellow dwarf virus-oat yellowing virus (BYDV-OYV). The isolate showed closest relationship with a new Chinese isolate (BYDV-PAV-CN), which has a genome of 5,652 nt (GenBank accession number: AY855920) (Bisnieks et al., 2004). However, comparison of the CP gene revealed that they shared only 87.8% amino acid identity (Liu et al., 2007). At the same time, this Chinese isolate showed only 71-74% amino acid identity with other isolates of BYDV-PAV (Jia et al., 2003) and it was 65% identical to BYDV-MAV isolates and 66% to BYDV-GAV (Liu et al., 2007).

2. Aims of the study

The objective of this study was to elucidate the relationship of BYDV-OYV, which is a new tentative species, to other BYDV species, using partial genome sequence analysis. Such information may support a better understanding of the molecular evolution of BYDV, the taxonomic status of BYDV-OYV and its relationship with BYDV-PAV-CN.

3. Materials and Methods

3.1. Plant materials

Plant materials previously shown to be infected with BYDV-OYV (Eriksson et al., unpublished) were used to obtain a partial viral genome sequence. In this study, virus-infected plant extracts were obtained from two different grasses from the county of Jämtland, Sweden, collected in 2010: couch grass (*Elytrigia repens*; Kvickrot) and *Festuca pratensis* (Ängssvingel).

In the current study, two extracts of *F. pratensis* and two extracts of *E. repens*, which had previously been positive in ELISA tests for BYDV-PAV and -MAV, were used. The CP gene was amplified by immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) (Bisnieks et al., 2004).

3.2. Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

Eppendorf tubes were coated with 50 µl polyclonal antibodies for BYDV-PAV (LOEWE Biochemica GmbH) and incubated overnight at 4ºC. After washing the tubes three times with 100 µl wash buffer (Table 2), 50 µl plant extract was added into each tube and the tubes were incubated overnight at 4ºC. After washing again (2 times with 100 µl wash buffer and 3 times with 100 µl Milli-Q water), reverse transcription (RT) and PCR were carried out (Liu et al., 2007; Malmstrom & Shu, 2004).

Table 2. ELISA buffers used in immunocapture

Coating buffer (pH 9.6; for 1000 ml distilled water)		**Washing buffer** (pH 7.4; for 1000 ml distilled water)	
Na_2CO_3	1.59 g	NaCl	8.00 g
$NaHCO_3$	2.93 g	KH_2PO_4	0.20 g
		$Na_2HPO_4(12H_2O)$	1.15 g
		KCl	0.20 g
		Tween 20	0.50 g

3.3. RT-PCR with CP gene

First, RT for the CP gene was carried out using primer Yan-R^{a} (Malmstrom & Shu, 2004). Yan-R^{a} primer (2 µl of 10 µM) and Milli-Q (9µl) water were added to the eppendorf tubes from step 3.2 where virus particles had been captured. The tubes were incubated at 70ºC for 10 minutes and rapidly cooled on ice for 5 minutes. Then, the cDNA synthesis was carried out using a cDNA synthesis kit (Superscript III Reverse Transcriptase, Invitrogen by Life Technologies) according to the manufacturer's instructions.

PCR amplification of the CP gene was carried out using the primer pair Yan-R^{a} and Shu-F (Table 3; Malmstrom & Shu, 2004) with 2 µl 10X Dream Taq buffer (Fermentas), 0.5 µl 10 mMdNTP, 0.12 µl Dream Taq, 15.5 µl distilled water and 2 µl cDNA. As a negative control, PCR was run without template ("water blanks") to monitor for contamination. The cDNA template of the CP gene was amplified using a T100^{TM} Thermal Cycler (Bio-Rad) with PCR conditions as shown in Table 4.

3.4. Agarose gel electrophoresis

The PCR products were analyzed in a 1% agarose gel with 0.5xTBE and stained with 1 µg/ml Gel Red. PCR product (8µl) or control PCR product was loaded into each lane and 3.5 µl of 80-10,000 bp Mass Ruler DNA Ladder Mix was used as size standard. After the electrophoresis was complete, the amplification products were visualized under ultraviolet light.

3.5. Gel extraction and purification

DNA fragments were purified from gels using a gel purification kit (Gene JET^{TM} Gel Extraction Kit, Fermentas) according to the manufacturer's instructions. For confirmation, purified PCR fragments were analyzed by gel electrophoresis.

3.6. Cloning of purified DNA

The purified DNA was cloned using CloneJET PCR Cloning Kit (Thermo Scientific) and Subcloning Efficiency *Escherichia coli* DH5α Competent Cells (Invitrogen) according to the manufacturers' instructions.

3.7. Colony PCR

The selected colonies were picked using 1-10 µl pipet tips and placed into 30 µl water in Eppendorf tubes. The presence of clone inserts was analyzed by gel electrophoresis after PCR amplification. Two µl 10X Dream Taq buffer (Fermentas), 0.2 µl pJET Forward sequencing primer, 0.2 µl pJET Reverse sequencing primer, 0.4 µl dNTP, 0.1 µl Dream Taq, 16 µl water and 1 µl bacterial suspension were used in the PCR amplification. The PCR amplification was carried out using the following program: initial denaturation at 95ºC for 2 minutes and 30 seconds, followed by 30 cycles of denaturation at 95ºC for 30 seconds, primer annealing at 50ºC for 30 seconds, primer extension at 72ºC for 1 minute and then final extension at 72ºC for 10 minutes.

3.8. Overnight bacterial culture

Clones containing inserts of the expected size were inoculated into 4 ml LB containing ampicillin 4 µg/ml and incubated overnight at 37ºC with shaking.

3.9. Purification of plasmid

Plasmid DNA was isolated from the overnight cultures using the Gene JET Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instructions. After purification, the yield and purity of plasmid DNA was measured using a nanodrop instrument (Thermo Scientific).

3.10. Restriction analysis of purified plasmid DNA and sequencing

The purified plasmids were cut by the two restriction enzymes *Xho*I and *Xba*I. For this procedure, 2 µl 10X Fast Digest Buffer, 0.5 µl *Xba*l, 0.5 µl *Xho*l, 16 µl water and 1 µl purified

plasmid DNA were used. The presence of inserts was analyzed and confirmed by electrophoresis. Twenty µl purified plasmid DNA (100 ng/µl) with insert of the expected size was sent to Macrogen Online Sequencing, Amsterdam, the Netherlands (http://www.macrogen.com/eng/) for sequencing. The purified plasmid DNA was sequenced using primers pJET forward and pJET reverse (Thermo Scientific).

3.11. Sequence analysis and virus-specific primer design

The obtained sequences were edited to remove sequences of primers and vectors before sequence similarity searches in the GenBank database with Blastn (http://www.ncbi.nlm.nih.gov/BLAST/). The determined sequences and the sequences from GenBank with highest identities were aligned using multiple sequence alignment, ClustalW (http://www.genome.jp/tools/clustalw/).

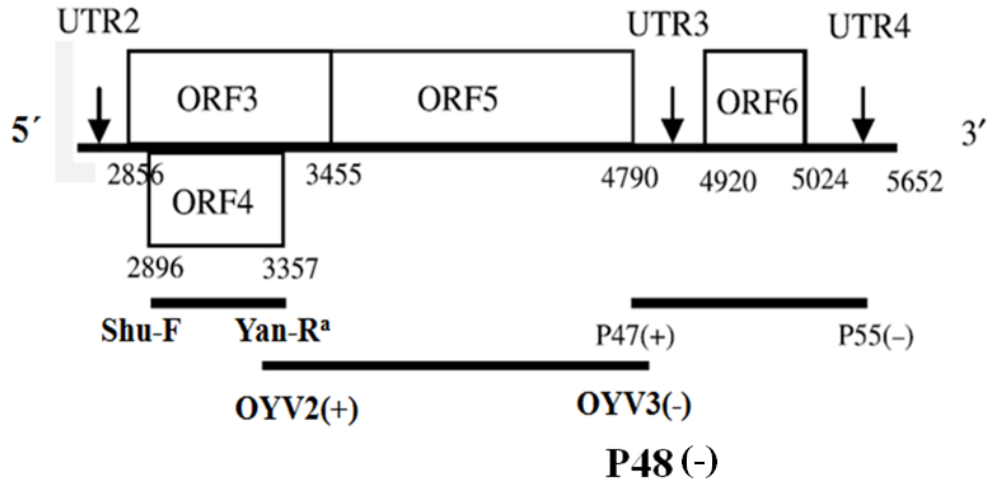

Fig. 2. Partial genomic map of members of the genus *Luteovirus* and localization of primers used (adapted from Liu et al., 2007).

To obtain additional sequence information for BYDV-OYV, previously published as well as newly designed primers were used (Fig. 2; Table 3). Two degenerate primers P47 (+) and P55 (-), which had been designed from the known BYDV-PAV-CN CP sequence (Liu et al., 2007),were used in PCR to amplify the ORF6 region of BYDV-OYV. Two universal primers, Yan-R^{a} and Shu-F (Malmstrom & Shu, 2004) were used to amplify the CP gene. Two primers, OYV2 and OYV3, were designed to obtain the ORF5 region. Primer OYV2 was

designed from a region of the CP gene and OYV3 was designed based on the obtained sequence of UTR3.

Table 3. Primers used in the experiment

Primers	Nucleotide sequences 5´ to 3´	Reference
Yan-R[a]	TGTTGAGGAGTCTACCTATTTG	Malmstrom & Shu, 2004
Shu-F	TACGGTAAGTGCCCAACTCC	Malmstrom & Shu, 2004
OYV2 (+)	TGAACTCGACACTGCGTGCA	Newly designed primer
OYV3 (-)	CTACCCGAGCTTATGAACCT	Newly designed primer
P47 (+)	GCAAAGGAGTACAAGGCACAAT	Liu et al., 2007
P48 (-)	GTTGACAGCCCACCCTCCAA	Liu et al., 2007
P55 (-)	GGATTGCTATGGTTTATGTCC	Liu et al., 2007

3.12. The partial nucleotide sequence of BYDV-OYV

3.12.1. Reverse transcription-polymerase chain reaction (IC-RT-PCR)

The virus RNA was captured using the immunocapture process like in step 3.2. RT was performed using different complementary sequence primers (Table 3) to synthesize cDNAs from different parts (Fig. 2) of the virus genome based on step 3.3. Then, the PCR amplifications of cDNAs were performed separately using different primer pairs and different PCR programs (Table 4) as explained in step 3.3.

Table 4. PCR conditions for primers used for amplification of the BYDV-OYV genome

Primer pairs	PCR conditions			Number of cycles
Yan-R[a]	Initial denaturation	95ºC	2.30 min	
Shu-F	Denaturation	95ºC	30 s	
	Annealing	55ºC	30 s	35

	Primer extension	72ºC	1 min	
	Final extension	72ºC	10 min	
	Indefinite hold	12ºC	∞	
OYV3 (-)	Initial denaturation	95ºC	2.30 min	
OYV2 (+)	Denaturation	95ºC	30 s	
	Annealing	53ºC	2 min	35
	Primer extension	72ºC	2 min	
	Final extension	72ºC	10 min	
	Indefinite hold	12ºC	∞	
P55 (-)	Initial denaturation	95ºC	2.30 min	
P47 (+)	Denaturation	95ºC	30 s	
	Annealing	48ºC	2 min	35
	Primer extension	72ºC	1 min	
	Final extension	72ºC	10 min	
	Indefinite hold	12ºC	∞	

After PCR amplifications, the same steps as mentioned in 3.4 to 3.10 were repeated to obtain the partial sequence of BYDV-OYV. The purified plasmid DNA of ORF6 was sequenced using primers pJET forward and pJET reverse (Thermo Scientific) like sequencing of CP gene. However, the purified plasmid of DNA of ORF5 was sequenced using primers T7 and SP6. ORF5 was amplified using sample Ängssvingel 30 instead of sample Ängssvingel 2, since Ängssvingel 2 was diluted to very low concentration.

3.13. Sequence and phylogenetic analysis

A partial genomic sequence of BYDV-OYV was assembled from the three sequenced PCR fragments using MegAlign (DNASTAR Lasergene 11) (Fig. 3). Two similar clones were used to obtain each fragment. The assembled sequence was analysed using ORF finder in NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/) to locate ORFs and untranslated intergenic regions (UTRs). The BYDV-OYV sequence was aligned with genome sequences of different B/CYDVs, which were obtained from GenBank (Table 5), using ClustalW in MEGA 5.2.1 (Tamura et al., 2011). The phylogenetic tree was constructed using the neighbor-joining algorithm in MEGA 5.2.1. Distances were calculated using Tamura 3-parameter model in the

analysis. The robustness of the internal branches of the tree was estimated by bootstrap analysis using 500 replicates.

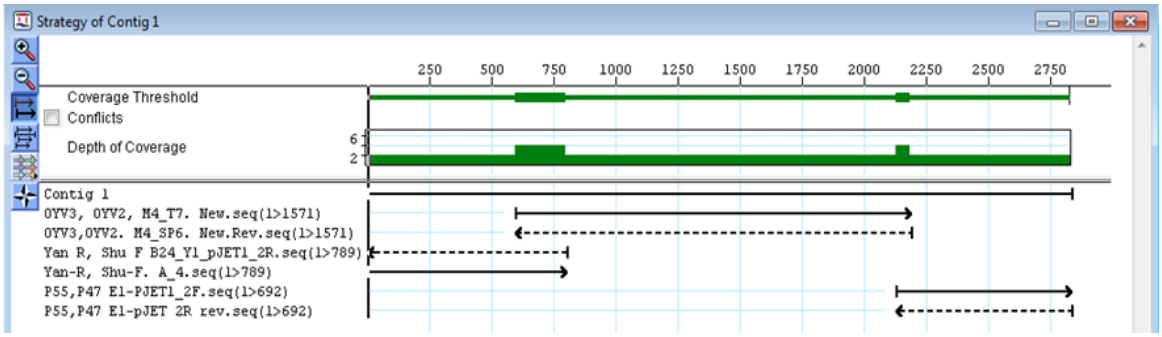

Fig. 3. Assembled sequence fragments of BYDV-OYV using MegAlign in DNASTAR.

Table 5. The viruses used in the alignment and phylogenetic analysis

Name of virus	Accession number
BYDV-PAS-129	AF218798
BYDV-PAV-III	AF235167
BYDV-GAV	AY220739
BYDV-PAV-CN	AY855920
BYDV-OYV	_____
BYDV-MAV-PS1	D11028
BYDV-PAV isolate 016	EF521835
BYDV-PAV isolate 052	EF521841
BYDV-PAV isolate 068	EF521846
BYDV-PAV isolate 05GG2	EU332309
BYDV-PAV isolate 05GG5	EU332310
BYDV-PAV isolate 05ZZ4	EU332321
BYDV-PAV isolate 05ZZ1	EU332320
BYDV-PAV isolate 06GY5	EU332329
BYDV-PAV isolate 06KM14	EU332332
CYDV-RPV	L25299
PAV-Sal1	AJ563410

4. Results

4.1. Selection of infected plant

In this study, plant materials previously shown to be infected with BYDV-OYV (Eriksson et al., unpublished) were used to obtain a partial genome sequence. The infected plant materials were obtained from two different grasses: *E. repens* (Kvickrot) and *F. pratensis* (Ängssvingel), from the county of Jämtland, Sweden in 2010. Initially, the CP gene was amplified by RT-PCR. Viral RNA was captured from BYDV-OYV infected leaves using immunocapture and then cDNA was obtained using reverse transcription. PCR amplification of the CP gene was carried out using the primer pair Yan-R[a] and Shu-F yielding a distinct DNA band of the expected size (around 790 bp) (Fig. 4). The gel picture indicates the successful amplification for (Ängssvingel-2) A2, (Ängssvingel-30) A30, (Kvickrot-K15) K-15 and (Kvickrot-K19) K19. No amplification was obtained in the negative control experiment indicating the absence of any contamination.

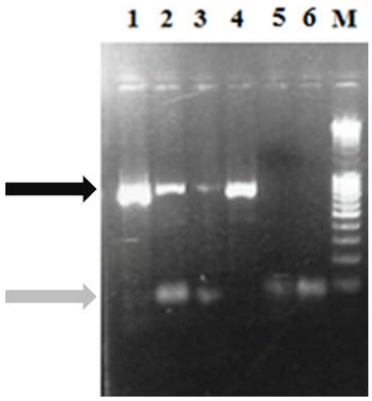

Fig. 4. PCR amplification products of the CP gene (~790 bp). Lanes 1 to 4 contain PCR products (1 – A2, 2 – A30, 3 – K15 and 4 – K 19) and lanes 5 and 6 contain negative control. Lane M contains 80-10,000 bp Mass Ruler DNA Ladder. The DNA bands marked with a black and gray arrow indicate expected size of PCR products and primer, respectively.

4.2. Gel extraction and purification of the CP gene

The PCR product of the CP gene for Ängssvingel-2 was purified using a gel extraction kit and then the purified DNA was run in a gel (Fig. 5). The purified DNA fragment showed the

expected size (~790 bp) and the strength of the band showed that the DNA amount was sufficient for cloning (Fig. 5).

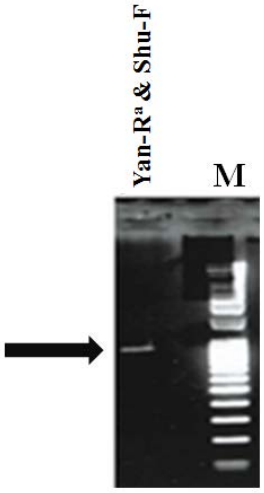

Fig. 5. PCR product for BYDV-OYV CP after gel purification. The purified DNA fragment had been amplified with the primers Yan-R^a and Shu-F (~790 bp) from the source plant Ängssvingel – 2.Lane M contains 80-10,000 bp Mass Ruler DNA Ladder. The arrow marks the expected size of purified DNA fragment.

4.3. Colony PCR

The purified DNA fragment was cloned into the pJET2.1 cloning vector in *E. coli* cells. Colony PCR was performed to select positive colonies, which contained the recombinant cloning vector. The expected DNA bands (~790 bp) were obtained for all tested colonies (Y1 to Y8) (Fig. 6) and four clones (Y1, Y3, Y4 and Y5) were used for subsequent bacterial culture.

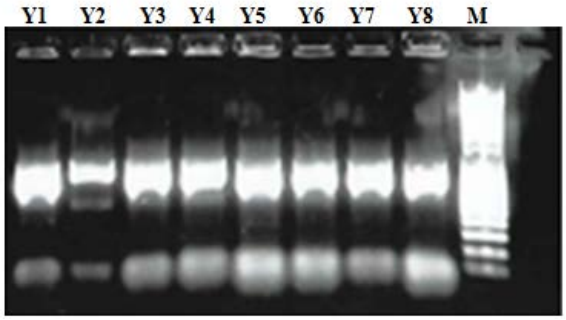

Fig. 6. ColonyPCR products for BYDY-OYV CP gene (insert size ~790 bp). M is 80-10,000 bp Mass Ruler DNA Ladder.

4.4. Restriction analysis of purified recombinant plasmids

After recombinant plasmid purification from overnight bacterial culture, the presence of correct insert was tested using restriction enzyme digests with *Xba*I and *Xho*I. The plasmids with inserts of the correct size were selected and sequenced. Gel electrophoresis showed that clones Y1, Y2 and Y3 contained inserts of the correct size (Fig. 7).

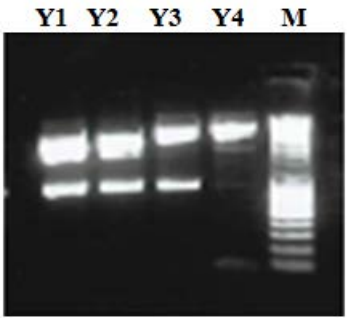

Fig. 7. Gel picture from plasmid restriction analysis with *Xba*I and *Xho*I to identity clones with insert of PCR product, which had been amplified using primers Yan-R[a] and Shu-F (insert size ~790 bp).M contains 80-10,000 bp Mass Ruler DNA Ladder.

4.5. RT-PCR with different sets of primers

First-strand cDNAs were synthesized from the viral genomic RNA using different complementary sequence primers (Table 3). Two different fragments of the BYDV-OYV genome (Fig. 2) were obtained using two different primer pairs (Table 3) in this process. One fragment mainly contained ORF5 encoding a readthrough protein and another one mostly contained ORF6 encoding the P6 protein.

According to the gel pictures (Fig. 8A & B), the amplified two different PCR products are of the expected sizes for the targeted genome regions of BYDV-OYV: ~1465 bp (ORF5) and ~690 bp (ORF6), respectively. The PCR product for ORF5 was obtained from the sample Ängsvingel-30 (A-30) (Fig. 8A) and for ORF6 (Fig. 8B) from Ängssvingel-2 (A-2).

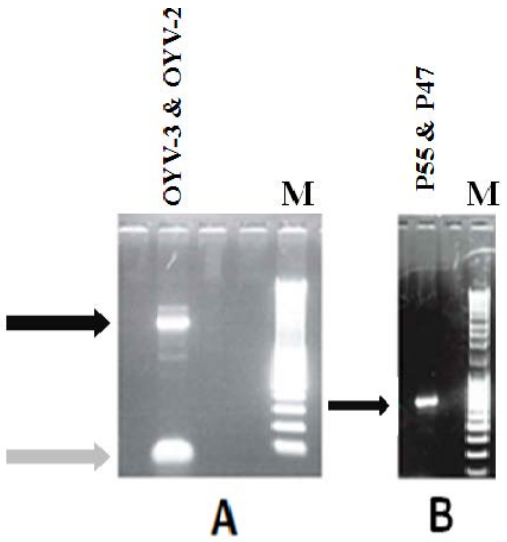

Fig. 8. PCR products for BYDV-OYV obtained with two sets of primers. A. The black arrow in figure A represents the PCR product, which was amplified using the primers OYV-3 and OYV-2 (~1465 bp), source plant Ängssvingel-30 (A-30). B. The black arrow in figure B indicates the DNA fragment was amplified using the primers P55 and P47 (~690 bp), source plant Ängssvingel-2 (A-2). Lane M contains 80-10,000 bp Mass Ruler DNA Ladder. The gray band shows primer dimer.

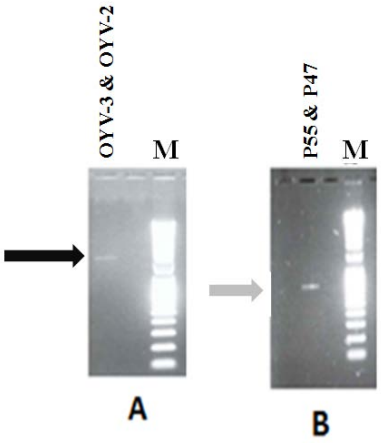

Fig. 9. PCR products for BYDV-OYV after gel purification. (A) The black arrow marks purified DNA fragment, which was amplified with the primers OYV-3 and OYV-2 (~1465 bp), source plant Ängssvingel-30 (A-30). (B) The gray arrow indicates amplification product with the primers P55 and P47 (~690 bp), source plant Ängssvingel-2 (A-2).Lane M contains 80-10,000 bp Mass Ruler DNA Ladder.

4.6. Cloning of PCR fragments (ORF5 and ORF6)

The purified DNA fragments (Fig. 9) were cloned into the pJET2.1 cloning vector in *E. coli* cells. Colony PCR was performed to select positive colonies, which contained the desired insert, but the expected DNA band was only obtained for the ORF6 clones. However, when the other fragment for ORF5 was cloned into the pGEM – T Easy Vector, a positive result was obtained.

4.7. Restriction analysis of purified recombinant plasmids (ORF5 and ORF6)

The restriction analysis of purified recombinant plasmids was performed to ensure that all the clones contained the expected DNA inserts. According to the analysis, clones O3 and O4 contained an insert of the expected size (~1465 bp) corresponding to the 3' ends of ORF4 and ORF3, and complete ORF5 (Fig. 10A). Clones P1 and P2 also showed positive results for cloning of a DNA fragment, which included the 3' end of ORF5, complete ORF6, complete UTR3 and partial UTR4 (Fig. 10B). The four positive clones (O3, O4, P1 and P2) were sent for sequencing.

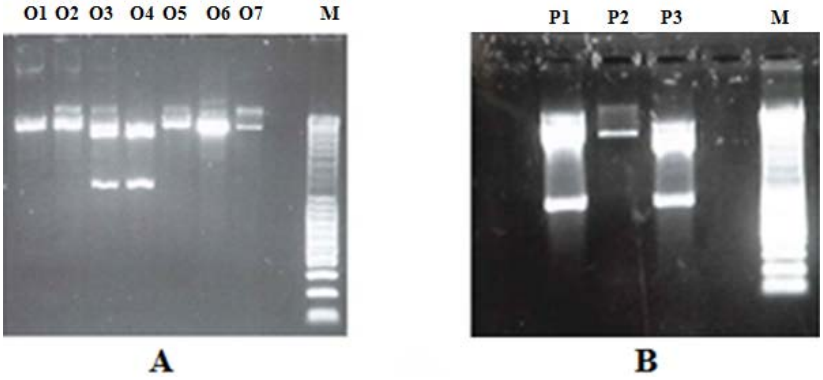

Fig. 10. Gel pictures from plasmid restriction analyses with *Xba*I and *Xho*I. (A) Cloning of PCR products amplified using primers OYV-3 and OYV-2 (insert size ~1465 bp). (B) Cloning of PCR products amplified using primers P55 and P47 (insert size ~690 bp). M is 80-10,000 bp Mass Ruler DNA Ladder.

4.8. Genomic organization of the partial BYDV-OYV sequence and comparisons with other members of the genus *Luteovirus*

Two similar clones for each of the three fragments were used in the analysis. The identity of the determined DNA sequences were confirmed by BLAST searches of GenBank. The confirmed sequences, which showed significant similarity to members of family *Luteoviridae*, were assembled using MegAlign (Fig. 3). The sequenced part of BYDV-OYV genome is 2,792ntlong and contained 4 ORFs and 3 UTRs (Fig. 11). The genome organization of the virus is similar to that of BYDV-PAV-CN (Liu et al., 2007) and other members of the genus *Luteovirus* (Fig. 1) (Domier, 2012). The sequenced genome region contains a partial UTR2 (193 nt). ORF3, 708 nt, encodes a coat protein with a calculated molecular weight (MW) of ~26 kDa. ORF4, 462 nt, encodes a putative movement protein of ~17 kDa and it is contained completely within ORF3, but in a different reading frame. ORF4 of the virus is similar to other members of family *Luteoviridae*. ORF5, 1260 nt, is translated in fusion with ORF3 as a result of in-frame translation readthrough of the ORF3 stop codon. ORF6, 123nt, of BYDV-OYV encodes a putative protein with a predicted MW of ~44kDa, which is similar to BYDV-PAV 05GG5 (EU332310) and BYDV-PAV-III (AF235167). UTR3 (79 nt) is located between ORF5 and ORF6 and varies in length among BYDV species. UTR4 is located downstream of ORF6.

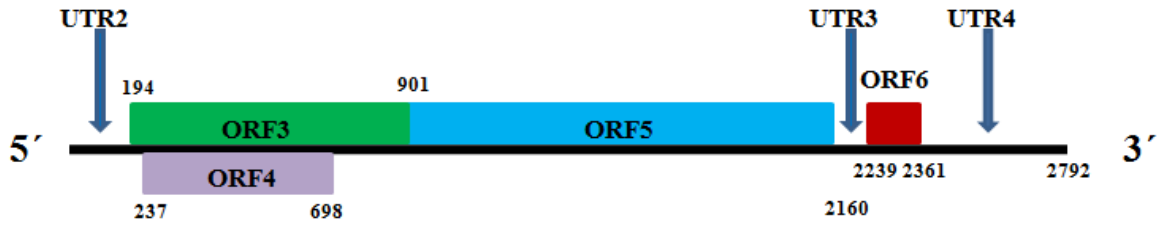

Fig. 11. Partial genomic map of BYDV-OYV.

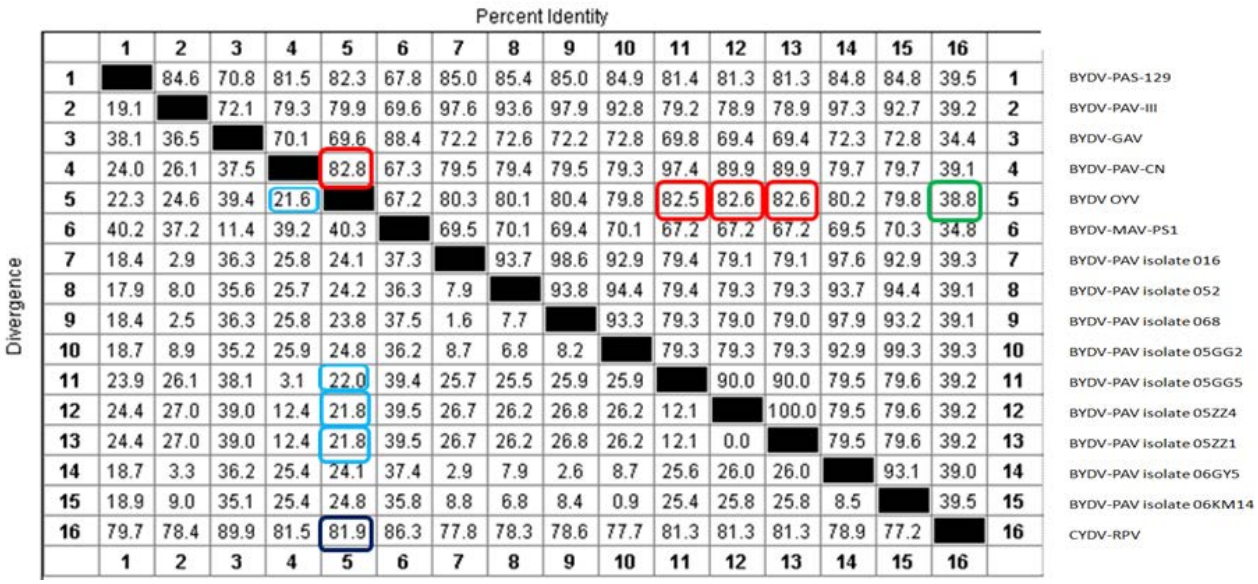

Fig. 12. Percent nucleotide identity between the partial genome sequence of BYDV-OYV (2,792 nt) and 15 isolates of family *Luteoviridae* (Table 5).

Among 15 analyzed Luteoviridae isolates, BYDV-OYV shared the highest nucleotide sequence identity with BYDV-PAV-CN (AY855920), BYDV-PAV isolate 05ZZ4 (EU332321), BYDV-PAV isolate 05ZZ1 (EU332320) and BYDV-PAV isolate 05GG5 (EU332310) at 82.8%, 82.6%, 82.6% and 82.5%, respectively (Fig. 12). Among these isolates, CYDV-RPV (L25299) showed lowest identity (38.8%) to BYDV-OYV.

4.9. Sequence comparisons of BYDV-OYV with other isolates of BYDV-PAV-CN and PAV-Sal1

Table 6. Percent nucleotide (nt) and deduced amino acid (aa) sequence identities between the CP gene (ORF3) of the Swedish BYDV-OYV isolate and available CP gene sequences of BYDV-PAV-CN and PAV-Sal1 (isolate of BYDV-OYV from Latvia)

Region	BYDV-PAV-CN		PAV-Sal1	
ORF3	nt	aa	nt	aa
	90	87	95	93

Comparisons were made between the CP sequences of the Swedish BYDV-OYV isolate and those of BYDV-PAV-CN and PAV-Sal1, which is an isolate of BYDV-OYV from Latvia (Bisnieks et al., 2004). In a previous study (Bisnieks et al., 2004), BYDV-PAV-CN and PAV-Sal1 showed the closest relationship, and they were clearly divergent from other isolates of PAV, PAS and MAV. The results showed that BYDV-OYV and PAV-Sal1 shared the highest sequence identities, with 95% and 93% nucleotide and deduced amino acid sequence identities, respectively. Comparison between BYDV-OYV and BYDV-PAV-CN showed that these two isolates shared lower nucleotide (90%) and deduced amino acid (87%) identities, respectively, compared with the identities between BYDV-OYV and PAV-Sal1.

4.10. Phylogenetic analysis

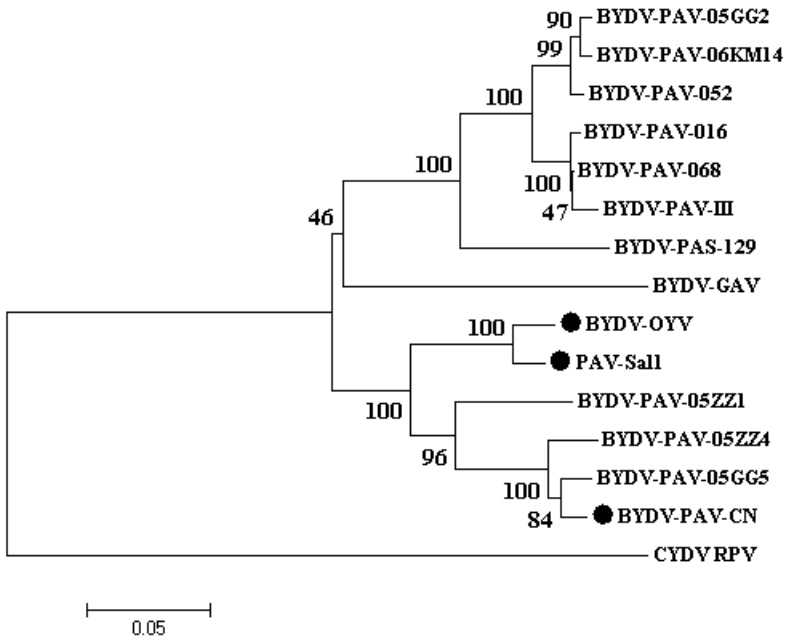

Fig. 13. Neighbour-joining phylogenetic tree constructed from the available CP gene of 14 members of the genera *Luteovirus* and *Polerovirus* and BYDY-OYV. The bootstrap values are indicated at the branch points. Horizontal lines are in proportion to the number of nucleotide differences between branch nodes. The scale bar corresponds to 0.05 estimated amino acid substitutions per site. Accession numbers are listed in Table 5.

A Neighbour-joining phylogenetic tree was produced to explain the relationship between the genera *Luteovirus* and *Polerovirus* and BYDY-OYV (Fig. 13) from available CP gene.

BYDV-OYV and Latvian isolate PAV-Sal1 are located in same clades. The location of BYDV-PAV-CN represents that the virus is distantly related to BYDV-OYV and PAV-CN.

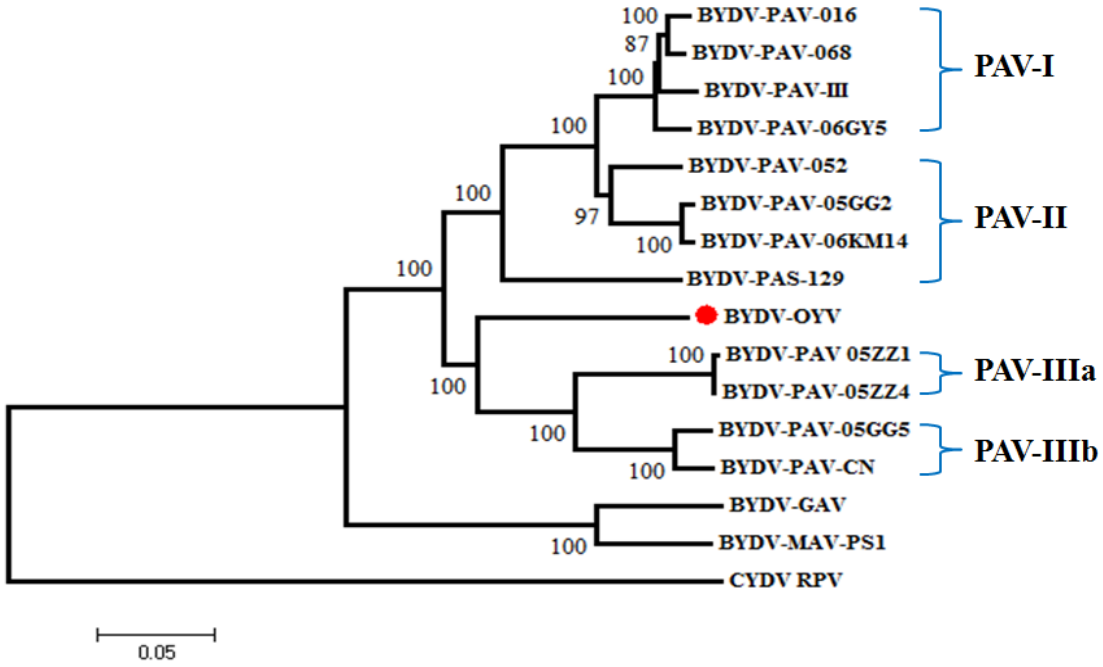

Fig. 14. Neighbour-joining analysis of 15 members of the genera *Luteovirus* and *Polerovirus* as well as BYDY-OYV based on the alignment of 2,792 nt of the partial genome. The bootstrap values are indicated at the branch points. Horizontal lines are in proportion to the number of nucleotide differences between branch nodes. The scale bar corresponds to 0.05 estimated amino acid substitutions per site. Accession numbers are listed in Table 5.

A phylogenetic tree was constructed based on partial nucleotide sequences of 15 isolates of the genera *Luteovirus* and *Polerovirus* as well as BYDY-OYV (Fig. 14). The tree contains two major significant clades and the species CYDV-RPV. CYDV-RPV is a polerovirus and forms an outgroup to the others, which are luteoviruses (genus *Luteovirus*). Among the luteoviruses, there are different well-supported groups (clades). BYDV-MAV and BYDV-GAV constitute one separate group (100% bootstrap). PAV-I (isolates of BYDV-PAV) and PAV-II (BYDY-PAS) form one group (100% bootstrap), among the PAV-like viruses, while BYDV-OYV, PAV-IIIa and PAV-IIIb form another group (100% bootstrap). According to the phylogenetic analysis, BYDV-OYV is most closely related to viruses of PAV-IIIa and PAV-IIIb, but it is still distinctly different.

5. Discussion

The primary aim of the study was to characterize and clarify the relationship of virus isolates of BYDV-OYV from the county of Jämtland, Sweden with other isolates of BYDV. BYDV-OYV is a new tentative species, which has been suggested in a study of BYDV diversity in Latvia based on CP gene sequence analysis (Bisnieks et al., 2004).The Latvian isolate was initially classified as BYDV-PAV (BYDV-PAV-Sal1) because it was positive for BYDV-PAV in ELISA and it was captured by BYDV-PAV antibodies in immunocapture. However, this classification turned out to be incorrect after sequence analysis of the RT-PCR product of the CP gene (Bisnieks et al., 2004).

The Swedish isolates of BYDV-OYV were also positive for BYDV-PAV in ELISA (Eriksson et al., unpublished).At the same time, a phylogenetic analysis with BYDV-OYV and 14 isolates of the genera *Luteovirus* and *Polerovirus* based on available CP gene sequences showed that BYDV-OYV was clearly separate from isolates of BYDV-PAV, BYDV-PAV-CN, BYDV-PAS and BYDV-MAV. The phylogenetic tree showed that the Swedish isolate of BYDV-OYV and PAV-Sal1 belong to the same species and they shared 95% nucleotide (nt) and 93% amino acid (aa) identities. According to the International Committee on the Taxonomy of Viruses (ICTV) guidelines, virus isolates with a variation at amino acid level exceeding 10% for any viral gene product could be classified as separate species within the family *Luteoviridae* (Domier, 2012). Bisnieks et al. (2004) have shown that the Latvian isolate PAV-Sal1 showed closest relationship with BYDV-PAV-CN, which frequently occurs in wheat in northern China (Liu et al., 2007). Nucleotide and deduced amino acid identities between the CP gene of BYDV-OYV and BYDV-PAV-CN proved that BYDV-OYV and BYDV-PAV-CN are different species with an aa sequence identity below 90% (Domier, 2012). They shared only 90% nt and 87% aa identities.

To understand better the relationships between BYDV-OYV, BYDV-PAV-CN and other species of family *Luteoviridae*, additional genomic sequences were amplified in this study. Some primers used in RT-PCR were the same as those used for obtaining the genome of BYDV-PAV-CN (Liu et al., 2007). Primer OYV3 (-) was designed to replace primer P48 (-) after difficulties to obtain the fragment covering ORF5. Primer OYV3 (-) was designed based on the sequence of the newly amplified ORF6 of BYDV-OYV. The reason for the failure of

amplification is probably because primer P48 (-) has the sequence TTGGA**G**GGTGGGCTGTCAAC and the corresponding region of the amplified UTR3 has a nucleotide difference at the 6th position (TTGGA**A**GGTGGGCTGTCAAC). This difference was discovered after amplification of the ORF6 fragment.

The phylogenetic analysis with the partial genome sequence of BYDV-OYV and other isolates of family *Luteoviridae* showed that BYDV-OYV belongs to family *Luteoviridae*. The position of BYDV-OYV showed that it is more closely related to the groups of PAV-IIIa and PAV-IIIb than to the groups of PAV-I and PAV-II (Liu et al., 2007; Wu et al., 2011). BYDV-OYV is grouping between PAV-I, PAV-II and PAV-III. Comparison of nucleotide identities between the partial genome sequence of BYDV-OYV (2,792 nt) and 15 isolates of family *Luteoviridae* also gave a similar result. BYDV-OYV shared a nucleotide identity of 82.8% with BYDV-PAV-CN (PAV-IIIb), 82.6% with BYDV-PAV isolate 05ZZ4 (PAV-IIIa) and BYDV-PAV isolate 05ZZ1 (PAV-IIIa) and 82.5% with BYDV-PAV isolate 05GG5 (PAV-IIIb). BYDV-OYV shows more than 10% nt difference and less than 90% nt identity to all other isolates of family *Luteoviridae*.

Although the findings with partial genome sequences support the hypothesis that BYDV-OYV is a new species within family *Luteoviridae*, a complete genome sequence of BYDV-OYV is necessary for a taxonomic classification. The complete genome sequence provides important information when classifying viruses according to the guidelines of the ICTV. For example, it became clear that BYDV-RMV is a new species in the genus *Polerovirus* and it was renamed *Maize yellow dwarf virus-RMV*(MYDV-RMV) after determining its complete genome sequence (Krueger et al., 2013). The BYDV-OYV genome sequence of the 3' part was amplified in the current study. However, the genome sequence of the 5' end is also important for generating a better understanding of the molecular evolutionary dynamics, which result from mutation, recombination and reassortment (Elena & Sanjuan, 2008; Wu et al., 2011). Combinations of these molecular events can produce new viral forms (Escriu et al., 2007). Recombination seems to be one of the strongest forces shaping the genome in plant RNA viruses, especially in family *Potyviridae*, which is the largest family of plant RNA viruses, but also in family *Luteoviridae* (Domier et al., 2002). It may lead to a dramatic change in the biological properties of the virus and play a role in the emergence of new viral pathogens, including resistance-breaking and host-switching strains (Sztuba-Solinska et al., 2011; Bujarski, 2013).

Determination of the complete genome sequence of an individual virus is also an important strategy to finding out an appropriate control strategy. The most environmentally feasible control strategy is breeding and the use of tolerant and resistant cultivars. Some natural resistance genes, such as the *Yd*2 gene in barley (Schaller et al., 1964) and *Bdv*1 in wheat (Makkouk & Kumari, 2009), have been identified against BYD-associated viruses. However, rapidly evolving viruses may evade these natural resistance genes very easily (Holmes, 2009; Garcia & McDonald, 2003; McDonald & Linde, 2002).

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