Oat sterile dwarf - A molecular method for determination of virus content in individual planthopper vectors

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1. Abstract

This study aimed to develop a method that would - in a relatively simple way - test a large number of individual planthoppers for virus content. This method would be a tool in trying to predict disease outbreak in the following year's crop.

The disease in question is Oat sterile dwarf, which can cause severe yield losses in oats in the middle area of Sweden. Oat sterile dwarf disease is caused by the *Oat sterile dwarf virus* (OSDV) and transmitted by a *Delphacidea, Javesella pellucida*. The disease has not caused noticeable yield losses since the 1960ies until the summer of 2001, when it hit again at one location. If disease outbreak tends to increase, then the ability to make accurate predictions will be appreciated.

OSDV is a fijivirus, genus *Reoviridae*, which are double-stranded RNA viruses. It replicates in its vector and in the plant host and is transmitted in a persistent manner by planthoppers. In Sweden, *J. pellucida* is the dominating vector. The virus remains in the insect when it moults, but is not passed on to eggs. The planthopper acquires and transmits the virus by feeding on infected cereals and grass.

The methods used for virus detection were reverse transcription polymerase chain reaction (RT-PCR) and dot-blot hybridisation. Primers for RT-PCR were developed, the fragments cloned and sequenced. The required sequences were compared to each other and to a published sequence of OSDV (Isogai *et al*., 1998). The sequenced fragments were used to synthesize a probe for hybridization.

Virus was detected in extracts of individual planthopper nymphs with RT-PCR and dot-blot hybridization and in oat plants with RT-PCR. Percentage of planthoppers containing virus range from 0-30 percent in the populations studied.

2. Svensk sammanfattning

I Lumsheden, på gränsen mellan Gävleborgs och Dalarnas län fick man sommaren år 2001 en kännbar påminnelse om ett problem som alla hoppades var gammalt och glömt. En lantbrukare fick där en havreskörd som var en tredjedel av den normala på hans mark. Havreplantorna var kraftigt bestockade och mycket kortväxta. Flertalet av vipporna var sterila och de få kärnor som utvecklats var små. På stråbaserna på vissa av de kortväxta plantorna syntes små gulvita svullnader. Detta var ett utseende som bara alltför väl kändes igen i denna del av Sverige, nämligen symptom på dvärgskottsjuka, eller ”Bollnässjuka” som det har kallats. På 1940 och -50-talen orsakade denna sjukdom stora skördeförluster hos havre i norra Svealand och södra Norrland. I början av 60-talet blev det något år med stora skördeförluster igen, men sedan dess har det varit ganska tyst om dvärgskottsjuka, så visst är det förståeligt att man trodde att faran var över för gott.

Dvärgskottsjuka i havre orsakas av ett virus som heter OSDV vilket står för *Oat sterile dwarf virus*. Viruset kan inte för egen maskin sprida sig mellan plantor, utan måste ta hjälp av en transportör, en vektor, i det här fallet en liten växtsugande insekt, den glasvingade ängsstriten (*Javesella pellucida*). Den glasvingade ängsstriten lever av växtsaft från gräs, gärna havre, och lägger sina ägg i grässtråna. Äggen kläcks på sommaren och den lilla vinglösa nymfen börjar suga på havreplantan. Nymferna kan hoppa en bit men de kan inte flyga bort från det fält där de kläckts. Striten genomgår fem nymfstadier innan den blir en vingad adult, och det är i det fjärde nymfstadiet den övervintrar. Under vintern befinner den sig i en lätt dvala, gärna vintergröna gräs, så om havren är skyddsgröda till en insådd vall är detta mycket gynnsamt för striten. Om havrefältet istället plöjs på hösten dör antagligen de allra flesta stritnymferna. I området kring Lumsheden är vall och havre populära grödor. De är väl anpassade till klimatet, och i takt med att nöt- och grisgårdarna försvunnit har kornodlingen minskat medan det är lätt att få avsättning för havre och hö till hästfoder.

Det verkar som om dvärgskottsjukan inte är ett helt utagerat problem. Även om sjukdomen bara slår till då och då är det intressant att veta mer om dess egenskaper. Jag ville försöka ta reda på varför sjukdomen har kommit tillbaka på vissa platser efter att ha varit i princip borta så länge. Vissa utredningar har gjorts om viruset och vektorn, och jag tänkte försöka bygga på den kunskap som redan finns. Odlingsteknik, växtföljd, sortval, temperaturvariationer mellan åren, och flera andra faktorer spelar antagligen också in. Genom att kombinera kunskaper om växten, viruset, vektorn och dessa abiotiska faktorer skulle det kanske i framtiden gå att förutspå ett angrepp av dvärgskottsjuka och förutsättningarna för att kunna vidta rätt åtgärder skulle förbättras.

En metod för att enkelt kunna testa hur stor andel av stritpopulationen som är virusförande skulle kunna förbättra en eventuell angreppsprognos. Försök har gjorts med serologiska tester (ELISA, *e*nzyme-*l*inked *i*mmunosorbent *a*ssay – specifika antikroppar producerade i en kanin eller häst binder till viruset och utlöser en färgreaktion) på strit- och växtmaterial men de har inte varit särskilt lyckosamma för just detta virus, och det finns inga antikroppar för viruset. Idag finns dock andra metoder, t ex PCR (*p*olymerase *c*hain *r*eaction). Att virustesta enskilda plantor och små grupper av stritar med PCR är fullt möjligt, men det är ändå en relativt dyr och tidskrävande metod om man vill testa ett stort antal. Därför skulle utvecklandet av en metod att med en enkel hybridisering av provet (strit eller växt) till en inmärkt probe

(komplementär bit av nukleinsyra som binder till ett specifikt ställe av virusets RNA) på ett nylonmembran ge en möjlighet att på kort tid testa ett stort antal prover (s.k. dot-blot).

Efter att virusets nukleinsyra har extraherats från växt- eller stritmaterial amplifieras (massförökas) en viss del av genomet med PCR. Denna del kan klonas i bakteriekulturer genom att den sätts in i en plasmid som tas upp av bakterierna. Det klonade materialets nukleinsyra sekvensbestäms och jämförs med tidigare sekvensbestämningar av viruset. Vid en sådan jämförelse framgår det säkert om det är rätt virus som isolerats. Den genetiska variationen hos olika isolat inom arten och mellan arter kan också bestämmas. De klonade fragmenten används också vid tillverkningen av den probe som används vid hybridiseringen. Proben är en komplementär DNA eller RNA sträng som binder specifikt till – idet här fallet virusets – genom. Proben märks in med en nukleotid som känns igen av en antikropp som har ett enzym bundet till sig. När enzymet bryter ned ett visst substrat sänd ljus ut. Vid hybridiseringen binds stritarna till nylonmembranet, proben tillsätts och tillåts hybridisera över en natt innan den tvättas bort från de ställen där den inte bundit. Substrat tillsätts och ljuset som sänds ut svärtar en film. Där proben har bundit (positiva prover som innehåller virus) blir mörka fläckar till skillnad från de prover där den inte bundit (negativa prover som inte innehåller virus).

I studien detekterades virus i enskilda stritar med både PCR och dot-blot hybridisering. Metoderna gav överensstämmande resultat. Ett fragment (709 nukleotider långt) av virusets genom, isolerat från både havre och strit, sekvenserades. Sekvenserna skiljde sig 1-2 % mellan de olika isolaten på nukleotidnivå.

1. Introduction

Oat sterile dwarf is a well-known disease in the middle area of Sweden where it was a severe problem in oats in the 1940 and -50 ties (Lindsten, 1970). In some parts oats was not grown for a period in order to stop the disease. It was stopped and was absent for a long period, until the summer of 2001 when a farmer discovered oat fields with typical symptoms of the disease that only produced one third of the normal yield of oats.

Extensive investigations where performed in the 1950 and -60 ties and it was concluded that Oat sterile dwarf was caused by a virus, *Oat sterile dwarf virus,* which is transferred by a insect vector. Vectors are all planthoppers (*Delphacidae*) and in Sweden *Javesella pellucida* is the most important vector for the disease.

2. Virus background

Viruses are nucleoproteins too small to be seen in a light microscope. They are all parasitic in cells and cause diseases in all forms of organisms, from microorganisms like bacteria, to insects, plants, and humans. They do not consume cells or produce toxins; they cause disease by utilizing and manipulating the cellular metabolism of the host (Agrios, 1997).

The viral genome has coding regions for proteins required for infection, movement within the plant, interaction with hosts, transport between hosts and a few non-coding regions that control the expression of the coding genes. Virus genomes are generally short, and they are efficiently used. There are no or very few introns (non-coding “junk-nucleotides”) that

eukaryote genomes often have a lot of, and some regions in the virus genome may even code for more than one protein with the use of different reading frames. The nucleic acid of viruses might be either DNA or RNA and both types could be either double or single stranded (Agrios, 1997).

4.1 Reoviridae

Members of the virus family *Reoviridae* have a genome of double stranded RNA, while most plant viruses have a single stranded RNA genome. This virus family consists of nine genera including viruses that infect plants, ticks, insects, fish, birds, and mammals (*Table* 1) (Uyeda and Milne, 1995). It is quite unique for virus families to have members that infect both plants and animals. *Human rotavirus* is a virus in the *Reoviridae* family that causes severe diarrhoea and the death of over 600.000 children annually in developing countries. Children in these countries may have a reduced immune defence due to undernourishment and may not get the clean water needed for rehydration during disease and hence are killed (Peter, 1997).

Genus	Type species	Hosts
Phytoreovirus	*Wound tumor virus*	Flowering plants, leafhoppers
Fijivirus	*Fiji disease virus*	Flowering plants, planthoppers
Oryzavirus	*Rice ragged stunt virus*	Flowering plants, planthoppers
Orthoreovirus	*Reovirus type 1*	Vertebrates
Orbivirus	*Bluetoungue virus*	Vertebrates, arthropods
Coltivirus	*Colorado tick fever virus*	Vertebrates, arthropods
Rotavirus	*Human rotavirus*	Mammals, birds
Aquareovirus	*Golden shiner virus*	Fish, cephalopods
Cypovirus	*Cytoplasmic polyderosis virus*	Insects

Table 1. Genera in the family *Reoviridae*. (Uyeda and Milne, 1995).

Plant viruses within the *Reoviridae* family are grouped into three genera, *Phytoreovirus, Fijivirus*, and *Oryzavirus* based on virion morphology, number and size of genomic segments and their electrophoretic profiles, serological relationship and insect vectors (Fang *et al.,* 2001). Insects from the families of planthoppers (*Delphacidae*) and leafhoppers (e. g. *Cicadellidae*) transmit plant reoviruses. All known plant reoviruses replicate in their vectors, and none is seed-borne or transmitted by mechanical means. They cause disease in plants, but are not, or only mildly, pathogenic to their insect hosts. This suggests that plant reoviruses originate from the insect vectors rather than from the plants (Noda and Nakashima, 1995).

4.2 Fijivirus

The genus *Fijivirus* are distributed worldwide except for North America. Members of the genus are *Fiji disease virus* (FDV), *Rice black-streaked dwarf virus* (RBSDV), *Maize rough dwarf virus* (MRDV), *Mal de Río Cuarto virus* (MRCV) (may be a South American strain of MRDV), *Pangola stunt virus* (PaSV), *Oat sterile dwarf virus* (OSDV) and *Nilaparvata lugens reovirus* (NLRV) (Marzachì *et al.,* 1995).

Fijiviruses are spherical particles, 65-70 nm in diameter. The particles have two shells with spikes on both shells (Milne *et al.,* 1974). The genome of fijiviruses is made up of 10 dsRNA

segments. The function of the proteins encoded by these segments has not been completely determined for any fijivirus so far, but for some proteins the functions are known.

The fijiviruses are divided into three serogroups (*Table* 2) based on lack of serological cross-activity among them (McMahon *et al.,* 1999). The type member of the genus, *Fiji disease virus* is the only member of serogroup one. FDV is a recently characterized virus that causes a long known disease in sugarcanes on Fiji and in parts of Australia and Oceania. OSDV is the only member of serogroup three and causes disease in *Poaceae* in central and northern Europe. The rest of the plant reoviruses are considered members of serogroup two, but it is not totally clear which ones should have species status and which ones should not. It is probable that some that are today considered distinct species are really geographical races of one species (Uyeda and Milne, 1995).

Serogroup	**Fijivirus**	**Location**
1	*Fiji disease virus*	Australia, Oceania
2	*Maize rough dwarf virus*	Mediterranean, South American
	Rice black streaked dwarf virus	South-east Asia
	Pangola stunt virus	Central America, Fiji, Taiwan, Northern Australia
3	*Oat sterile dwarf virus*	North and central Europe

Table 2. Fijiviruses (Uyeda and Milne, 1995).

NLRV is a non-plant pathogenic member of the genus. The leafhopper *Nilaparvata lugens* occurs in South-east Asia, where it damages rice by feeding and by acting as a vector for two other viruses (*Rice ragged stunt virus*, RRSV, an oryzavirus and *Rice grassy stunt tenuivirus*) that damage the rice plants. NLRV has been detected in almost all organs of its hopper host, with the highest levels in the fat body, salivary glands and the intestine (Noda and Nakashima, 1995).For two fijiviruses, NLRV (Nakashima *et. al*., 1996) and RBSDV (Zhang *et al.,* 2001), the entire genome has been sequenced.

4.3 Oat sterile dwarf fijivirus

OSDV is the only member of serogroup 3 of the *Fijivirus* genus. The virus infects *Poaceae* in Europe and its natural vectors are planthoppers (*Delphacidae*) (Lindsten,1970). *Arrhenatherum blue dwarf virus* and *Lolium enation virus* found in *Arrhenatherum eliatus* and *Lolium multiflorum,* respectively, are considered forms of OSDV (Milne and Lesemann, 1978).

In a Swedish isolate of OSDV the four smallest segments (segment 7, 8, 9 and 10) of the 10 genome segments have been sequenced and the functions of the proteins investigated (Isogai *et al*., 1998). In this project, the sequence of OSDV segment 8 was used when finding out primers for reverse transcription and polymerase chain reaction. OSDV segment 8 probably encodes an outer shell protein.

5. Oat sterile dwarf disease

OSDV is the cause of a severe disease in oats (*Avena sativa*), the Oat sterile dwarf disease.

5.1 Disease host range

The virus infects members of the *Poaceae* family, and disease occurs in some species, but symptoms tend to be less severe than in oats. In 1977, Milne and Lovisolo tested 23 members of the *Poaceae* family for virus susceptibility. 16 species out of 23 were found susceptible and 7 were found not to be susceptible (*Table* 3). All non-*Poaceae* species are also insusceptible.

Susceptible hosts	**Svenskt namn**	**Insusceptible hosts**	**Svenskt namn**
Arrhenatherum elatius	Knylhavre	*Agrostis gigantea*	Storven
Avena fatua	Flyghavre	*Alopecurus myosuroides*	Renkavle
Avena sativa	Havre	*Apera spica-venti*	Åkerven
Cynosurus cristatus	Kamäxing	*Bromus inermis*	Foderlosta
Festuca pratensis	Ängssvingel	*Digitaria sanguinalis*	Blodhirs
Holcus lanatus	Luddtåtel	*Echinochloa crus-galli*	Hönshirs
Hordeum vulgare	Korn	*Elymus repens*	Kvickrot
Lagurus ovatus	Harsvans		
Lolium multiflorum	Ital. rajgräs		
Lolium perenne	Eng. Rajgräs		
Lolium temelentum	Dårrepe		
Phalaris canariensis	Kanariegräs		
Poa annua	Vitgröe		
Secale cereale	Råg		
Triticum aestivum	Vete		
Zea mays	Majs		

Table 3. Hosts and non-hosts of Oat sterile dwarf disease after Milne and Lovisolo (1977).

5.2 Geographic distribution

Disease is found in the former Czechoslovakia, Finland, Germany, Norway, Poland, Sweden and the UK (Lindsten, 1970).

5.3 Symptoms of disease

Symptoms of Oat sterile dwarf disease are abnormal formation of side shoots, stunted shoots, few and small kernels, dark green color and sometimes enations, or vein swellings, on shoot bases (*Figure* 1) (Lindsten, 1970).

Figure 1. Symptoms of Oat sterile dwarf disease.
Healthy plant to the left.

5.4 Transmission

OSDV is transmitted by an insect vector. It cannot be transmitted by contact between plants, by seed or by pollen. Insect vectors are planthoppers from the *Delphacidae* family: *Javesella pellucida, J. dubia, J. discolor, J. obscurella* and *Dicantropis hamata*, with *J. pellucida* (in Swedish: Glasvingad ängsstrit) as the principal natural vector (Lindsten 1970).

6. Biology of *Javesella pellucida*

6.1 Nymphal stages and over-wintering

J. pellucida has five nymphal instars before the adult stage. The nymphs have undeveloped wings and can only move a few meters by walking or jumping. In Scandinavia, *J. pellucida* over-winters mainly in the third or fourth nymphal stage and only one generation per year is developed, while in central Europe, e.g. Germany, two generations per year are developed (Raatikainen, 1967).

As egg-laying preferably takes place in spring cereals, over-wintering usually takes place in winter green grasslands close to these fields or in unploughed fields. In Sweden, most eggs hatch in August, and the first to third or fourth nymphal instars develop in the cereals and on the stubble in autumn. If the cereal or grass they feed on is infected with virus, the nymphs can become infected and are then infectious for the rest of their lives. The third or fourth to fifth instar is developed in spring the coming year.

6.2 Adult stage and egg-laying

Two forms of the adult *J. pellucida* occur: the brachypterous or short-winged form, and the macropterous or long-winged form. In natural populations of *J. pellucida*, the macropterous form dominates greatly, and the rate is further increased by crowding (Ammar, 1973; Mochida, 1973). The macropterous forms fly at heights of 2-6 meters and can migrate several kilometers. In Sweden, the adults start to migrate to new fields with spring cereals in May or June depending on temperature, and there mating and oviposition take place (Lindsten, 1970). Females lay between 268 and 437 eggs each during their average 38 to 48 days as adults (Ammar, 1975).

Oviposition usually takes place into the stems of spring cereals. The incubation time of the eggs varies with temperature, but is in Finland and Sweden, on average, four weeks (Raatikainen, 1967).

6.3 Population densities of Javesella pellucida

J. pellucida is the most frequently occurring *Delphacidae* in agricultural land in the middle area of Sweden and Finland, and the dominance of the species averages 63% of the total *Delphacidae* fauna. Population densities of *J. pellucida* vary between years and during the season, as shown by Raatikainen (1967) The number of specimens peaked at the turn of June-July all four years (1959-1962) that Raatikainen investigated these species (*Table* 4).

Year	**Weather during growing season**	**Specimens caught at peak**
1959	Warm and dry	1312
1960	Very warm, moderately wet	442
1961	Average warmth and wetness	50
1962	Very cool, wet	120

Table 4. Specimens of *J. pellucida* caught with 200 sweeps of a sweep net at the turn of June-July, four years with different weather during the growing season (Raatikainen 1967).

7. Aims of study

The aim of this project was to create and evaluate a method for testing a relatively large number of planthoppers (*J. pellucida*) individually for virus content. This method could then be coupled to population densities, abiotic factors (weather etc.) and rates of disease outbreak; to create a disease forecast method and possibilities to design preventive measures.

8. Material and methods

8.1 Sources of material

Plant and insect material was mainly collected in the summer of 2002. Numbers refer to samples for Reverse transcription and Polymerase chain reaction (RT-PCR), see Results.

1. Oat plants from Gävle, collected in 2002.
2. Oat plants from Stocksbo, Lumsheden, on the border between Dalarna and Gästrikland. Collected in 1987. These plants have symptoms of OSDV. Plants have been stored at –20°C.
3. Planthoppers (*Javesella pellucida*) from the same location as number 2, collected in 2002. Planthoppers were collected with sweep net and stored at room temperature in 75% ethanol.
4. Oat plants from the same location as 2 and 3 collected in 2001. Plants were stored at +4°C.

Nymphs and adult planthoppers for hybridization were also collected at other locations in the counties of Västmanland, Dalarna, Gävleborg and Uppsala (*Table* 5). Adults were trapped in yellow water traps and nymphs with a sweep-net in oat fields. They were counted weekly during the summer 2002 in order to calculate changes in population densities during the growing season.

8.2 Extraction of total genome

Samples of plant tissue (0.1 g) or planthoppers (0.03 g = 20 planthoppers) were ground in liquid nitrogen with a mortar and pestle. Total RNA and DNA was extracted, including the double stranded RNA (dsRNA) genome of the virus, mainly as described by Déley and Corio-Costet (1998), which in brief is an extraction with chloroform and isopropanol.

8.3 Primers for Reverse transcription and Polymerase chain reaction

In order to design appropriate primers for the RT-PCR (reverse transcription and polymerase chain reaction) the sequences of the OSDV segments determined by Isogai *et al.* (1998) were used. The sequences of OSDV were compared to those of other species of fijiviruses in order to find segments that had the same nucleotide sequence in more than one species (conserved regions). These regions were hard to find, as the different species of fijiviruses are quite different from each other genetically. Finally two primer pairs were chosen from segment 8. The primers are each 20 nucleotides long and called OSDV 1 (bp 20-39 on segment 8 according to Isogai *et al.,* 1998), OSDV 2 (bp 98-117), OSDV 3 (bp 662-681) and OSDV 4 (bp 827-846).

OSDV 1: 5' ATG GAA GAC CTA CAT ATC AA 3'

OSDV 2: 5' TTA AAT GAC CGC CCA ACA AT 3'

OSDV 3: 5' TTA TAA TCA TAA CCT CCA TC 3'

OSDV 4: 5' TCC AAT ACT GAC AGT TCT TC 3'

The fragments that are amplified with primer 2 and primer 3 are 544 nucleotides and with primer 2 and primer 4 they are 709 nucleotides long (primers not included).

8.4 Reverse Transcription and Polymerase Chain Reaction

By use of the enzyme reverse transcriptase, a complementary DNA strand (cDNA) that can be used for PCR was synthesized. Reverse transcriptase uses RNA as a template to synthesize cDNA.

1µl of plant or insect extract was mixed with 2µl of 10 µM primer OSDV 4 and 7 µl dH_2O. The nucleic acids were denatured by 5 min incubation at 100°C and then immediately moved to ice and incubated there for 15 min. To each sample was added 4 µl 5 x reaction buffer, 2µl 0.1M DTT, 1µl 10mM dNTP, 1 µl Rnasin and the samples were incubated at 37°C for 2 min before 1 µl of 200u/µl reverse transcriptase (Fermentas, Lithuania) was added. Samples were incubated at 37°C for 1h for reverse transcription to take place and then diluted 1:5 with dH_2O to 120µl.

For PCR, 5µl of this dilution was mixed with 1µl of 10 µM primer 2, 1µl of10 µM primer 3 or primer 4, 5 µl 10 x reaction buffer containing $MgCl_2$, 1 µl 10 mM dNTP, 0.7 µl Taq-polymerase (Expand High Fidelity PCR system kit; Roche, Switzerland) and 36.3 µl dH_2O. As two pairs of primers (2&3 and 2&4) were used on each sample, there were in total 8 reactons and 2 negative controls with no sample added, only water.

Conditions for PCR:

Pre-heating 2 min at 94°C,

35 cycles of:
94°C for 30 s (denaturing)
47°C for 60 s (annealing)
72°C for 2 min (elongation)

Post-heating at 72°C for 10 min.

After reverse transcription and PCR, the PCR fragments were checked on a gel and purified with the QIAquick PCR Purification Kit (Qiagen, Germany).

8.5 Ligation and cloning

The PCR fragments were ligated into a pGEM-T Easy vector using the pGEM-T Easy Vector System kit (Promega, USA). The pGEM-T Easy vector is a 3015 nucleotides long *Escherichia coli* plasmid that carries genes for ampicillin resistance (*amp*^{r}) and the *lacZ'* gene. By using a selective agar medium (ampicillin added to Luria-Bertani (LB) medium) only transformed cells can form colonies. This is an important selection as only a minority of the competent cells is transformed.

The *lacZ'* gene makes it possible to distinguish cell colonies with recombinant plasmids from those where the plasmid has self-ligated. The DNA fragment is inserted so that it inactivates the *lacZ'* gene (insertional inactivation). Without an insert, the *lacZ'* gene encodes an enzyme (β-galactosidase) that digests lactose and breaks it down to glucose and galactose. The same enzyme also breaks down a lactose analogue that is called X-gal (5-bromo-4-chloro-3-inolyl-β-D-galactopyranoside) to a product that is bright blue. When X-gal is added to the agar, cell colonies with a self-ligated plasmid will become blue while cells with a recombinant plasmid remain white. It is then easy to identify transformants (Lac selection) and multiply these in overnight cultures using liquid LB medium (Brown, 2001).

The pGEM-T Easy vector also has recognition sites for SP6 and T7 RNA polymerases. The promoter sequences are positioned on either side of the insert site on the plasmid and make transcription with either polymerase possible.

"Subcloning Efficiency DH5α Competent Cells" (Invitrogen, USA) of *E. coli* were used for cloning.

8.6 Preparation of plasmid DNA

Plasmid DNA was prepared from the transformed cells with the "QIAprep Spin Miniprep Kit" (Qiagen). The cells were lysed under alkaline conditions that denature DNA. Lowering of the pH caused precipitation of chromosomal DNA while plasmid DNA stayed in the supernatant. The lysate was cleared by centrifugation. Plasmid DNA from the supernatant was absorbed to a silica membrane in a micro-column, while RNA and proteins passed through. Salts were washed away and finally the plasmid DNA was eluted.

8.7 Sequencing of cloned inserts

The insert size was checked by digestion with the restriction enzyme *Eco*RI.

The absorbance of the samples was measured on an atom absorption spectrophotometer and the concentration was calculated:

C (μg/ml) = Abs (260 nm) x times diluted x constant (50 for DNA) x length of cuvette

For the sequence analysis 0.5 μg DNA was used together with the "DYEnamic ET terminator cycle sequencing kit" (Amersham Life science, USA) and an ABI Prism 377 DNA Sequencer (Perkin –Elmer Applied Biosystem, USA). The inserts were sequenced with M13 forward and M13 reverse primers. Four clones of the longer insert (709 bp) were completely sequenced in both directions with OSDV 3 and a new primer (OSDV 5, bp 350-369 on segment 8) localized 250 bp downstream of primer OSDV 2.

OSDV 5: 5' GAG GTT AAT CAA CCA TCA AT 3'

The shorter fragment (544 bp) was also sequenced. The acquired sequences were compared to each other and the previously sequenced Swedish isolate (Isogai *et al.,* 1998) using BLAST 2 (Altschul *et al*., 1990) and Clustal W (Thompson *et al*., 1994) programs. Phylogenetic analyses were carried out using the PAUP software, version 4 (Swofford, 2002). The distances for the neighbor–joining analysis were calculated using the Kimura two-parameter model. A parsimony analysis was also done.

8.8 Restriction with NcoI *and* NdeI

To avoid run-around transcripts of the vector and the insert, linearization of the plasmid with clone 2d (containing the short, 544 nucleotides, insert) was done by digestion with *Sal*I, *Nco*I or *Nde*I, which do not cut in the insert and have unique sites in the polylinker region of the vector. The restriction sites for *Sal*I and *Nde*I are located at the 3'side of the insert and the site for *Nco*I is located at the 5'side. All three of them cut with a 5'overhang, which is preferred for the *in vitro* transcription.

After a test restriction the templates were analyzed on a gel, and as *Nde*I and *Nco*I gave the best result, they were used for restriction of the rest of clone 2d. Proteins and other contaminants that could disturb the *in vitro* transcription were removed by extracting the template twice with phenol/chloroform and once with chloroform only.

8.9 In vitro *transcription*

DIG-labeled probes were produced by *in vitro* transcription of an OSDV fragment. Digoxigenin (DIG) is a steroid that has been linked to uridine nucleotides and can be identified by an anti-DIG antibody. Digitalis plants (*Digitalis purpurea,* Swedish: Fingerborgsblomma) are the only natural sources of digoxigenin, so the anti-DIG antibody does not bind to other biological material (van Miltenburg *et al.,* 1995).

The two templates (2d cut with *Nde*I or *Nco*I, respectively) were mixed with dNTP labeling mix, transcription buffer, RNase inhibitor, dH_2O and SP6 or T7 RNA polymerase (Roche). The *Nde*I template was used with SP6 RNA polymerase and the *Nco*I template was used with T7 RNA polymerase. The transcription took place during incubation at 37^oC for 4 h. After 2 h of incubation, a gel was run to check the transcription.

8.10 Preparation of samples for hybridization

Samples	Number of Samples
Nymphs and adults from Lumsheden, Dalarnas/Gävleborgs län	45
Nymphs from Kågbo, Gävleborgs län	10
Nymphs from Lindesberg, Örebro län	10
Adults from Uppsala län	2
Plants from three locations	3
Total	70

Table 5. Samples for hybridization.

Planthoppers (*J. pellucida*, stored in 75% ethanol) were ground individually with a pestle in a 1.5 ml microcentrifuge tube with 25 µl Tris-Cl 10mM, pH 8.5 (Polston *et al*., 1990). Two individuals of leafhoppers (*Psammotettix alienus*, Swedish: Randig dvärgstrit) was treated in the same way as *J. pellucida* and used as negative controls. 10 µg/ml DNA fragments from the RT-PCR (sample 3) was diluted 10, 100, 1 000 and 10 000 times (concentrations: 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml and 0,001 µg/ml respectively) and used as positive controls and as markers for detection level and for estimation of virus concentration in the planthoppers. On the membranes, 5.0 - 12.5 µl of each sample (avoiding insect parts) was spotted onto a Hybond-N nylon membrane (Pharmacia, USA) and allowed to be absorbed for a few minutes. The membrane was carefully moved using rounded forceps to denaturing solution (1.5M NaCl, 0.5M NaOH) for five minutes, avoiding the samples to be washed away. Then the membrane was moved to a neutralizing solution (1.5M NaCl, 0.5M TrisHCl, pH 7.5) for five minutes and finally moved to a washing solution (2 x SSC) for five minutes. Access solution was allowed to drip of and the damp membrane was placed on a sheet of damp (dipped in 2 x SSC) Whatman paper and treated for 60 sec in an UV oven (Amersham Life science, USA) to make the nucleic acids bind covalently to the membrane. The membrane was allowed to air dry and was then stored in a folded Whatman paper and wrapped in aluminum foil at room temperature.

8.11 Prehybridization and Hybridization

The membrane and a mesh of appropriate size were prewetted in 2 x SSC solution, rolled up and put into a hybridization tube. 15 ml of prehybridization solution (50% deionzed formamide, 5 x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent) was added to the tube. It was then allowed to prehybridize rotating for 1h at 65^{o}C in a hybridization oven. After 1h the probe was added to the hybridization solution in the tube (van Miltenburg *et al.,* 1995). The probe was denatured in water bath for 10 min at 65^{o}C. The kit manual suggested 20-100 ng of probe/ml hybridization solution. The *Nco*I probe has an estimated concentration of 100 µg/ml. 2 µl of the probe was added, and was allowed to hybridize rotating at 65^{o}C overnight (20 ng probe / ml hybridization solution).

8.12 Detection

After hybridization the membrane was washed in the hybridization oven with warm (65^{o}C) washing solutions 0.1 – 2 x SSC + 0.1% SDS. The concentration of salt (SSC) in the washing solution was gradually decreased to increase the stringency of the washing: two washes of 15 min with 2 x SSC + 0.1% SDS rotation at +65^{o}C and one wash of 15 min with 0.1 x SSC + 0.1% SDS on shaker in room temperature with warm (+65^{o}C) washing solution that was gradually cooling down. The membrane was equilibrated in washing buffer (100mM maleic acid, 150mM NaCl, pH 7.5, 0.3% Tween 20) for 1 min before blocking in blocking solution (100mM maleic acid, 150mM NaCl, pH 7.5, 1% skim milk) in a dish that was slowly agitated for 1h. Anti – Digoxigenin - AP (antibodies) were diluted 1:10 000 in blocking solution near the end of the blocking period, and the membrane was moved to this dilution and left there for 30 min. The antibody solution was discarded and the membrane washed twice in washing buffer. The membrane was equilibrated for 2 min in detection buffer (100mM Tris-HCl, 100mM NaCl; pH 9.5) and entirely soaked in CSPD (substrate) diluted in 1:100 detection solution. After letting excess liquid to drip off, but not letting the membrane to get dry, the membrane was sealed into a plastic bag to keep it moist and protected. The membrane was exposed to X-ray films for 4 min to 44 h before developing.

8.13 RT-PCR of individual planthoppers

To verify the hybridization results, the extracts of positive samples from the hybridization tests and some of the negative ones were used for RT-PCR. No further extraction or purification of the ground planthoppers was done, and RT-PCR was carried out as described above. Primers OSDV 2 and OSDV 3 were used for this RT-PCR.

9. Results

9.1 Samples for RT-PCR

Total nucleic acids, including double-stranded RNA, were extracted from oat plants and planthoppers for RT-PCR with phenol/chloroform (Délye and Corio-Costet, 1998). This extraction method was originally designated for fungi. The extracts were run on a test-gel and the nucleic acids seemed to be in good condition except in the refrigerated plant-material (sample nr 3, *Table* 6) where it was degraded (not shown). Virus was detected with RT-PCR in the oat plant collected in 1987 (sample 2 and 7) and the planthoppers collected in 2002 (sample 3 and 8, *Table* 6), while the other samples were negative (*Figure* 2).

Sample	Primers OSDV 2 and OSDV 3	Sample	Primers OSDV 2 and OSDV 4
1	Oat plant collected in 2002	6	Oat plant collected in 2002
2	Oat plant collected in 1987	7	Oat plant collected in 1987
3	Planthoppers collected in 2002	8	Planthoppers collected in 2002
4	Oat plant collected in 2001	9	Oat plant collected in 2001
5	Negative control primer 2 & 3	10	Negative control primer 2 & 4

Table 6. Samples for RT-PCR.

Both pairs of primers gave fragments of expected size for the positive samples as the bands on the test-gel show (*Figure* 2.). The short fragment (primers 2 and 3, sample 2 and 3) is 544 nucleotides and the long fragment (primers 2 and 4, sample 7 and 8) is 709 nucleotides long.

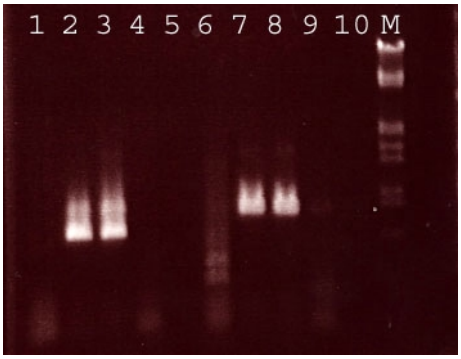

Figure 2. PCR samples and size marker (M)
Numbering of samples as in table 6.

9.2 Sequences of isolates

Sequencing of cloned fragments of the virus genome showed that the genetic variation between the 15 clones sequenced was 1-2% on nucleotide level. Virus isolated from plant or insects did not differ more than different clones from the same virus source. The variation between clones sequenced in this project and the Swedish OSDV clone sequenced by Isogai *et al.* (1998) is also 1-2%.

9.3 Parsimony analysis

A parsimony analysis and a neighbour-joining analysis was done, comparing one acquired sequence from this project (709 bp fragment isolated from planthopper) with the published OSDV isolate and three other fijiviruses (*Figure* 3). The results of the parsimony analysis were identical to those using neighbour-joining. For RBSDV two isolates are in the analysis, Zeihang and Wuhan (*Table* 7). The parsimony analysis constructs a hypothetical evolution from an ancient ancestor that is common for all isolates. The horizontal lines are proportional to the number of changes that has occurred from this common ancestor, on amino acid level. The structure of the parsimony tree was validated with a bootstrap analysis. Bootstrap values are shown on the horizontal lines in the figure. The analysis shows that the two OSDV clones compared are closely related to each other while they are distantly related the other fijiviruses.

Fijivirus	Segment	Accession number	Source
OSDV	8	AB011025	Isogai *et al.*, 1998
RBSDV	10	AJ297433 Zeihang	Zhang *et al.,* 2001
RBSDV	10	AJ291707 Wuhan	Zhang *et al.,* 2001
MRDV	10	L76561	Marachì *et al.,* 1996
NLRV	8	D26127	Nakashima *et al*., 1994

Table 7. Origins of fijivirus clones in the parsimony analysis.

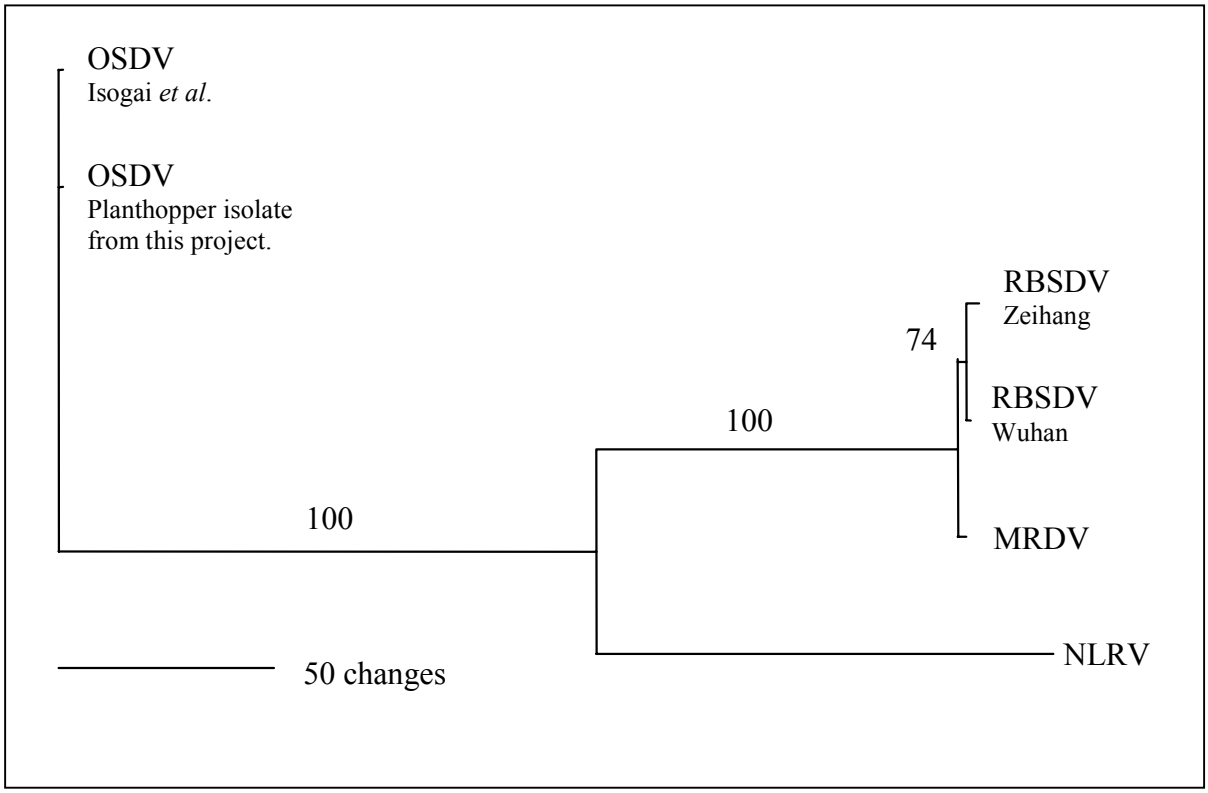

Figure 3. Parsimony analysis. The length of the horizontal lines indicates the number of changes during evolution from the common ancestor. The shorter the line, the closer the relationship between isolates. Bootstrap values are shown on horizontal lines.

9.4 Virus detection by hybridization

Virus in individual *J. pellucida* adults and nymphs was detected by hybridization with the DIG-labeled probe (*Figure* 4*)*. Virus was detected in nymphs and in adult planthoppers, while results from plant samples were difficult to analyse, with light dots instead of nothing for the negative samples (*Figure 5*).

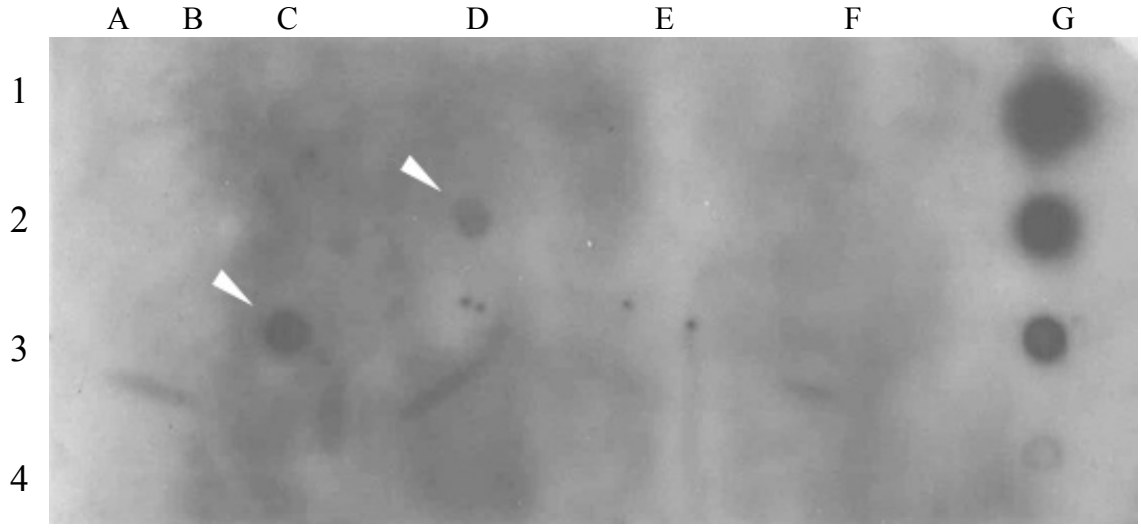

Figure 4. Hybridization membrane with two positive samples (white arrows). A1 to D3 are adult planthoppers from Lumsheden. D4 and E1 are adults from Uppsala. E2 to E4 are nymphs from Lumsheden. F2 and F3 are negative controls. G1 to G4 are positive controls, RT- PCR fragments diluted (from top) x 10, x 100, x 1.000 and, x 10.000, respectively.

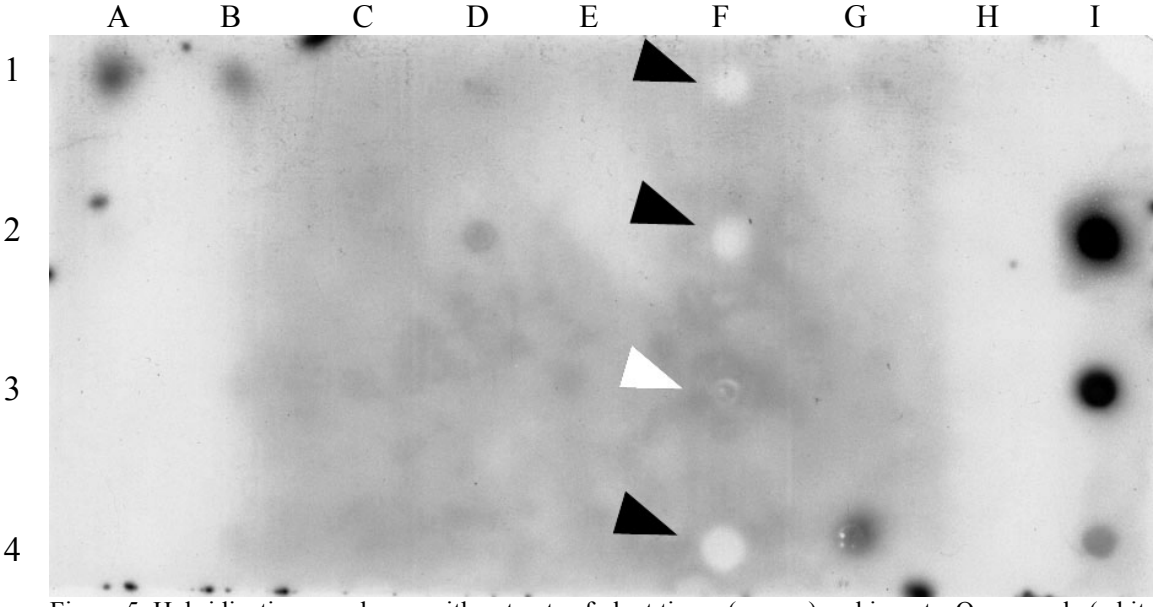

Figure 5. Hybridization membrane with extracts of plant tissue (arrows) and insects. One sample (white arrow) is known to be infected with virus, but results from hybridization are unclear. A1 to C2 are nymphs from Kågbo. C3 to E4 are nymphs from Lindesberg. F1 to F4 are extracts of oat plants.G1-H3 are nymphs from Lumsheden. H4 is a negative control and I2 to I4 are positive controls (RT-PCR fragments).

9.5 RT-PCR on individual planthoppers

RT-PCR was also done on individual planthoppers to verify the results from the hybridization. The only extraction to produce this extract for RT-PCR was grinding individual planthoppers in 10 mM Tris x Cl. RT-PCR was done on the two positive samples from the first hybridization membrane and three samples that were negative from that membrane. The same results were obtained with RT-PCR as with hybridization for these samples (not shown).

9.6 Infected planthoppers

The proportion of planthoppers infected with virus varies between 0 and 30 percent in the populations examined (*Table* 8.). As only a small number of insects have been tested from each population, the statistical accuracy of these percentages is uncertain. At Kågbo (30 percent infected planthoppers) symptoms of disease has not been observed.

Population Site	**Nr. Samples**	**Nr. Positive**	**% Positive**
Lumsheden, Dalarnas/Gävleborgs län	45	6	13
Kågbo, Gävleborgs län	10	3	30
Lindesberg, Örebro län	10	2	20
Upplands län	2	0	0
Total	67	11	16

Table 8. Total number of planthoppers tested and proportion infected.

10. Discussion

For most plant diseases there are many factors, rather than just one, that affect disease development. OSDV in oats is probably no exception to this rule. Annual variations in climate, plant development and over-wintering conditions for the planthopper vectors will probably influence disease rate in infected areas to a large extent. For plant diseases caused by a virus, the infection rate in the vector populations may also contribute to variations in disease outbreak and severity (Agrios, 1997). This study aimed to develop a method for testing the infection rate of OSDV in populations of *J. pellucida* planthoppers on a relatively large-scale basis. Testing of individual planthoppers in different populations of *J. pellucida* would contribute to the understanding of the disease, its fluctuations, and conditions affecting its spread.

If occurrence and severity of the disease expand to the level of 1950-1965, it would be important to be able to predict an expected disease outbreak in the following year's spring crop and if needed take preventive measures. To be able to carry out such a prediction, as many as possible of the involved factors must be considered. The *J. pellucida* nymphs could be tested for virus infection during autumn or winter and the infection rate of next years populations would be known. The results of testing must be considered together with other factors and coupled to the actual degree of disease outbreak for several years before acceptable predictions can be made.

Genetic variation between the two isolates of OSDV sequenced in this study, as well as the published OSDV sequence, is only 1-2% on nucleotide level. The same low degree of genetic variation within the species is found in Chinese isolates of RBSDV which differs 0-2.3% on nucleotide level (Bai *et al.*, 2002).The compared OSDV isolates date from three years, 1987, 1994 and 2002, and are isolated from plants as well as insect vectors, still the genetic variation is small. Variation between different species of fijiviruses is large, OSDV isolates differ approx. 35% from RBSDV and MRDV on nucleotide level, while FDV differs approx. 30% and NLRV 60% from OSDV (Bai *et al.*, 2002).

Virus was found in populations of *J. pellucida* also at locations where the disease has been absent in recent years. Crop management and growing conditions at these locations would be interesting to study in order to understand why no disease outbreak has occurred. This would help us design pest management measures other than use of insecticides.

Levels of virus infected planthoppers varied between 0-30 percent in the populations studied, but more planthoppers need to be tested to obtain statistically reliable results.

The methods used in this study for detection of virus content in individual planthoppers (Dot-blot Dig-hybridization and RT-PCR) are more sensitive than e.g. ELISA (Hull 2002) Results were also congruous between the two methods. Still, nothing is known about the infected insects' ability to transfer virus to plants under field conditions. This ability may not correlate to the detection level of virus in insects. This must be tested in coupled field, greenhouse, and molecular experiments.

The method with Dig-labeled probe hybridized to virus in extract of planthoppers on a nylon membrane is less time and cost consuming than RT-PCR, once the probe is constructed. This

is especially true if many samples are to be tested at the same time. If many hundreds of samples will be tested, grinding of individual insects for hybridization will also be time consuming, and other attempts may be tried e.g. there are ways to statistically estimate the infection rate samples with more than one insect per sample. By using this method several, e.g. 10, insects are ground together and dotted on the membrane as one sample.

A few samples of plant tissue were tested on the hybridization membrane. One was known to contain OSDV (from RT-PCR) but results were not clear on the membrane. It appeared that something in the plant tissues blocked the membrane so that the probe could not bind. If plants are to be tested with this method a more careful extraction could improve the results by removing lignin, proteins, and other compounds that may block the membrane.

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