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Comparison of the Nucleotide Sequences of *Wheat Dwarf Virus* (WDV) isolates from Hungary and Ukraine

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Abstract

Wheat dwarf virus (WDV) is the most ubiquitous virus in cereals causing huge losses in both Hungary and Ukraine. The presence of barley- and wheat-adapted strains has been confirmed, suggesting that the barley strain is restricted to barley, while the wheat strain is present in both wheat and barley plants. Five WDV isolates from wheat plants sampled in Hungary and Ukraine were sequenced and compared with known WDV isolates from GenBank. Four WDV isolates belonged to the wheat strain. Our results indicate that WDV-Odessa is an isolate of special interest since it has originated from wheat, but belongs to the barley-adapted strain, providing novel data on WDV biology and raising issues of pathogen epidemiology.

Key words: *Wheat dwarf virus* (WDV), nucleotide sequence of WDV

Introduction

During the last decade *Wheat dwarf virus* (WDV) has been the most frequently isolated and most ubiquitous cereal-infecting virus in Hungary and it has now become a serious problem also in the Ukraine (Mesterházy *et al.*, 2002, Szunics *et al.*, 2003, Snihur *et al.*, 2007). WDV is a frequent causal agent of dwarfing, mottling, yellowing or reddening in cereals and suppressed heading and root growth in infected plants can drastically reduce yield. WDV was first described by Vacke (1961) in the former Czechoslovakia and subsequently found in Sweden (Lindsten *et al.*, 1970), Bulgaria (Stephanov and Dimov, 1981), Hungary (Bisztray *et al.*, 1989), France (Lindsten and Lindsten, 1993), Germany (Huth, 2000), Poland (Jezewska, 2001), Finland (Lemmetty and Huusela-Veistola, 2005), Romania (Jilaveanu and Vacke, 1995), Spain (Achon *et al.*, 2006), Tunisia (Najar *et al.*, 2000), Turkey (Köklü *et al.*, 2007), Zambia (Kapooria and Ndunguru, 2004), Ukraine (for the first time approximately in 1975 (Razvyazkina 1975), then in 2007 (Snihur *et al.*, 2007) and China (Xie *et al.*, 2007). WDV is transmitted by the European grass-feeding leafhopper *Psammotettix alienus* (Vacke, 1961) in a circulative, non-propagative manner (Lindsten and Vacke, 1991), therefore the occurrence of diseased plants in the field depends on the presence of the vector. During the crop screening for WDV conducted in 2009–2010, the unique virus vector, *Psammotettix alienus*, has been found in abundance in Ukrainian agroecosystems, providing indirect proof of widespread presence of WDV in the Ukraine.

WDV belongs to the genus *Mastrevirus* (family *Geminiviridae*) infecting monocotyledonous plants. Mastreviruses have a monopartite single-stranded genome of circular DNA and the genome encodes four different proteins: movement protein (MP) and coat protein (CP) on the viral sense strand, and two replication-associated proteins (Rep and RepA) on the complementary strand (Gutierrez, 1999). The presence of an intron in the Rep gene makes it possible for WDV to produce two different forms of the replication protein. The non-coding long intergenic region (LIR) and short intergenic region (SIR) contain sequence elements necessary for viral replication and transcription. The LIR comprises the origin of rolling circle replication of the virus (Heyraud *et al.*, 1993). The SIR contains polyadenylation signals and a region to which a short complementary primer for the second strand synthesis binds (Kammann *et al.*, 1991).

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Two different forms of WDV exist: a wheat-adapted form (WDV wheat strain) and a barley-adapted form (WDV barley strain) (Lindsten and Vacke, 1991; Bendahmane *et al.*, 1995, Kvarnheden *et al.*, 2002). Both strains infect a number of plant species in the family *Poaceae* (Lindsten and Vacke, 1991). There are, however, contradictory reports on whether the wheat strain can infect barley, and whether the barley strain can infect wheat. Lindsten and Vacke (1991) and Tóbiás *et al.* (2009) observed no transmission of the barley strain to wheat, whereas the wheat strain was transmissible to barley. Similar conclusion was made by Kundu *et al.* (2009), who found that the barley strain is restricted to the barley host, while the wheat strain is present in both wheat and barley plants. Mechner *et al.* (2003) detected both WDV strains in barley plants in the fields, using strain specific primers. Commandeur and Huth (1999) and Schubert *et al.* (2007) found that the barley strain can infect wheat only under laboratory conditions.

The genomes of the barley and wheat strains of WDV share an average of 85% identity. The isolates within the wheat strain show a high degree of homology (>98% identity), whereas the isolates of the barley strain are more variable (>94% identity). As the demarcation criterion for mastrevirus species has been set to 75% nucleotide sequence identity by the International Committee for Taxonomy of Viruses (Fauquet *et al.*, 2008), both strains are currently considered to belong to the same species. Schubert *et al.* (2007) recently proposed two new mastrevirus species *Barley dwarf virus* (BDV) and *Oat dwarf virus* (ODV) based on DNA sequence differences. ODV was accepted as a new tentative mastrevirus species sharing 70% genome-wide nucleotide sequence identity with the wheat and barley strains of WDV (Fauquet *et al.*, 2008).

The aim of the present study was the molecular characterisation of WDV isolates from Hungary and Ukraine and their comparison with the available sequences of WDV.

Experimental

Materials and Methods

Virus isolates. Symptoms of viral infection were found during spring observations carried out in wheat crops in Martonvasar (Middle Hungary), Pula (Southern Hungary) and Mironivka (Middle Ukraine). Plants displaying yellowing of leaves or dwarfing were placed in an insect-proof greenhouse and were tested for WDV by ELISA using a WDV kit (Bio-Rad). The collected WDV-infected plants were replanted into clay pots and placed in an insect-proof isolation net. For virus transmission, thirty individuals of virus-free *Psammotettix alienus* Dahlb. were placed underneath each net. One week later the leafhoppers were transferred to young seedlings of wheat being in two leaves stage. Six weeks later the plants were tested again for WDV by ELISA. Three isolates, WDV-HU-2Marton (collected in 2008 from Martonvasar), WDV-HU-Pula

Table I
Primers used for sequencing

Name	Genome position[1]	Sequence (5'-3')
WDV-Barley forw	468–488 (B)	ATCCCGGGTCCTCCGACTAC
WDV-Barley rev	478–458 (B)	GACCCGGGATCGTAAGGGGC
WDV-Barley 540	555–531 (B)	TAAGCCAAACAAACAACTCCTACGG
WDV-P1	611–631 (B)	GACCGAGGAAATTGGTTACGG
WDV-5'	1045–1067 (B)	CCACTGACATCTTTACGATGCC
WDV-Barley 1200	1200–1225 (B)	AACTACGTAGTGGGGAAGAATATCG
WDV-Barley 1900	1895–1917 (B)	CATAGGTCGTGAAATTCAACTAG
WDV-Barley 2110	2094–2122 (B)	TTCGAGGCTTACGGAGTAGAGATGTTCAT
WDV-Wheat P1	475–494 (W)	GACCGAGGAAATTGGTTACGG
WDV-Wheat 483	506–485 (W)	GCTTATACACAGCCCCCTTCC
WDV-Wheat 5'	809–831 (W)	CCACTGACATCTTTACGATGCC
WDV-Wheat 1076	1069–1087 (W)	TAAGAAAGGAGCACTGTATC
WDV-Wheat 1410	1428–1406 (W)	GCGAGTCATTCATCAACTACTCG
WDV-Wheat 1850	1850–1482 (W)	CCACTCCTGCGGATCAAGC
WDV-Wheat forw	2305–2326 (W)	ACGAAGCTTGTTCTGCACGAGA
WDV-Wheat rev	2316–2295 (W)	AACAAGCTTCGTGCTTCCATC
WDV-Wheat 2521	2521–2542 (W)	CAGAAGTCCGGCAGGTCCTTA

[1] With reference to WDV-Heves (FM999833) – B and WDV-2 Marton (FN806785) – W.

Table II
Abbreviation, accession number and origin of *Wheat dwarf virus* isolates used in this study

Abbreviation	Accession number	Country of collection
WDV-HU-B	AM040732	Hungary
WDV-HU-F	AM040733	Hungary
WDV-HU-H07	FM210034	Hungary
WDV-HU-Heves	FM999833	Hungary
WDV-HU-Dunakiliti	FM999832	Hungary
WDV-HU-Martonbar	AM747816	Hungary
WDV-HU-2Marton	FN806785	Hungary
WDV-HU-Pula	FN806786	Hungary
WDV-Uk-g	FN806783	Ukraine
WDV-Uk-Miron	FN806784	Ukraine
WDV-Uk-Odessa	FN806787	Ukraine
WDV-BU-Bg17	AM989927	Bulgaria
WDV-Swe-Enk1	AJ311031	Sweden
WDV-Swe-Enk2	AM491490	Sweden
WDV-Swe-SE	X02869	Sweden
WDV-Chi-hbsjz061	EF536870	China
WDV-Chi-ynkm062	EF536881	China
WDV-Chi-sxyl052	EF536878	China
WDV-Chi-gsgg050	EF5368591	China
WDV-Chi-sxyl051	EF536877	China
WDV-Ge-SxA22	AM296022	Germany
WDV-Ge-SxA23	AM296023	Germany
WDV-Ge-SxA24	AM296024	Germany
WDV-Ge-SxA25	AM296025	Germany
WDV-Ge-SCBB21	AM296021	Germany
WDV-Ge-BaW1	AM411651	Germany
WDV-Ge-BaW2	AM411652	Germany
WDV-Ge-McP20	AM296020	Germany
WDV-Ge-Sx18	AM296018	Germany
WDV-Cz-6217	FJ546189	Czech Republic
WDV-Cz-6239	FJ546190	Czech Republic
WDV-Cz-W	FJ546188	Czech Republic
WDV-Cz-1841	FJ546191	Czech Republic
WDV-Cz-19	AM296019	Czech Republic
WDV-Cz-11105	FJ546180	Czech Republic
WDV-Cz-8100	FJ546179	Czech Republic
WDV-Cz-11229	FJ546181	Czech Republic
WDV-Cz-6482	FJ546178	Czech Republic
WDV-Cz-B	FJ546193	Czech Republic
WDV-Tr-bar	AJ783960	Turkey

WDV isolates sequenced in this study are indicated in bold type.

(collected in 2007 from Pula) and WDV-Uk-Miron (collected in 2009 from Mironivka and maintained in our greenhouse by subsequent transmission) were selected for further studies. Ten wheat samples from the Odessa region (South Ukraine) and one from Glevakha (Central North Ukraine) were initially tested by PCR with WDV specific primers. Two samples (WDV-Uk-Odessa and WDV-Uk-g collected from Odessa and Glevakha, respectively) were selected for molecular characterization.

Isolation of virus DNA, cloning and sequence analysis of the WDV isolates. DNA extraction was done according to Shepherd *et al.* (2008) with a slight modification (fresh leaf material was used instead of dry leaves). The samples were then stored at –20°C or used directly as a template for rolling circle amplification (RCA) of the WDV genome (Haible *et al.*, 2006). One microliter of the final Extract-n-Amp DNA solution was mixed with 4 µl of Templi Phi™ sample buffer (TempliPhi™, Amersham Biosciences), heated for 2 min at 94°C, and then brought to room temperature. Five µl of reaction buffer and 0.2 µl of enzyme mix were added to the cooled mixture and the Templi Phi™ extension reaction was run at 30°C for 18–20 h. WDV genome concatemers (multiple copies of unit-length virus genomes covalently linked end-to-end) generated during Phi29 DNA polymerase amplification were digested with *Hind*III (wheat strain) or *Sma*I (barley strain) to release unit-length genomes. After digestion genomic DNA was separated in 1% agarose gel and extracted with a DNA purification kit (Fermentas DNA Extraction Kit). The WDV genome was inserted into a *Hind*III or *Sma*I digested pBSK+ plasmid (Stratagene). The recombinant plasmids were transformed into *Escherichia coli* DH5α (Sambrook *et al.*, 1989).

Clones containing inserts with the expected size of 2.7 kb were sequenced with the DyeDeoxyTerminator Kit (Applied Biosystems) using reverse, universal (–20) and internal primers (Table I). Sequence analysis was performed using University of Wisconsin Genetics Computer Groups (GCG) sequence analysis software package version 9.1.

In order to determine the phylogenetic relationships between different WDV isolates complete genomes were analysed (Table II). Sequence alignment, tree formation, and bootstrap analysis were done with the help of the software Clustal X 1.83.

Results and Discussion

This work has been focused on the screening of Hungarian and Ukrainian cereal ecosystems for the presence of *Wheat dwarf virus* and its unique vector, *P. alienus*. The outcomes of the 2-year monitoring clearly demonstrated significant spread of the virus in Ukraine and confirmed the positive tendency in its spread compared to previous years of observations. In addition, for the first time a virus vector has been

Table III
Sequence identity of complete genomes of the WDV isolates characterized in our laboratory

WDV	B	F	2Marton	H07	Heves	Dunakiliti	Bg17	Mironivka	.g	Odessa
Pula	99.5	99.5	99.2	85.3	85.5	85.3	85.4	98.7	99.5	85.5
B		99.6	99.4	85.1	85.3	85.1	85.2	98.7	99.6	85.3
F			99.3	85.2	85.4	85.2	85.3	98.7	99.4	85.3
2Marton				85.3	85.2	85	85.1	98.4	99.3	85.2
H07					99.3	99	96.3	84.9	84.9	96.5
Heves						99.4	96.6	85.1	85.1	96.8
Dunakiliti							96.6	84.9	84.9	96.9
Bg17								85.1	85	99.3
Mironivka									98.7	85.2
.g										85.1

Abbrevations and accession numbers: WDV-HU-B: AM040732, WDV-HU-F: AM040733, WDV-HU-H07: FM210034, WDV-HU-Heves: FM999833, WDV-HU-Dunakiliti: FM999832, WDV-BU-Bg17: AM989927, WDV-Uk-g: FN806783, WDV-Uk-Miron: FN806784, WDV-HU-2Marton: FN806785, WDV-HU-Pula: FN806786 and WDV-Uk-Odessa: FN806787

shown to be common to Ukrainian fields since it has been detected in virtually every region of the country where the virus was identified.

Depending on the place of origin, the degree of WDV infection of collected wheat plants was 20–70% as confirmed by ELISA tests and PCR. In laboratory, the virus was transmitted by *P. alienus* in insect-proof isolation net and maintained on oat or wheat plants. Nucleic acids were isolated from plants infected with WDV-HU-2Marton, WDV-HU-Pula, WDV-Uk-g and WDV-Uk-Odessa, WDV-Uk-g and WDV-Uk-Miron and used for subsequent molecular and phylogenetic characterization.

The Extraction-n-Amp DNA extraction method was very rapid and simple in order to isolate intact viral DNA. WDV genome amplification *via* the RCA method generates large DNA concameters, from which unit-length genome were subsequently cleaved with *Hind*III or *Sma*I enzymes. The size of the full length genome obtained for WDV-HU-2Marton and WDV-HU-Pula constituted 2750 nucleotides, while the genomes of WDV-Uk-g and WDV-Uk-Miron were 2749-nucleotide-long, and the WDV-Uk-Odessa genome was exactly 2734 nucleotides in length. The very special property of this latter isolate is hat it has originated from the winter wheat variety Selyanka. To our knowledge, this is the first report of the barley strain of WDV isolated from naturally infected wheat plants. The genomes of these characterized isolates contained all four expressed mastrevirus ORFs (MP, CP, Rep, RepA), and the intergenic regions LIR and SIR.

The nucleotide sequences were deposited in GenBank as WDV-Uk-g: FN806783, WDV-Uk-Miron: FN806784, WDV-HU-2Marton: FN806785, WDV-HU-Pula: FN806786 and WDV-Uk-Odessa: FN806787, and were further compared to previously characterized WDV isolates (Tobias *et al.*, 2006, 2009, 2010) (Table III).

The analysis of the full genome sequences revealed high levels of identity among wheat strains and higher level of diversity among barley strains. The sequences' identities between isolates of the wheat strain of different geographical origins were very similar (>98.7% identity). For the movement protein (MP) and coat protein (CP), we observed high sequence identity (>98.8%) at the predicted amino acid level, in some cases MPs (Hungarian and Swedish isolates) and CPs (Hungarian isolates originating from different parts of the country) were identical. For the short (SIR) and large intergenic region (LIR), we observed a higher variability (97% and 96.6% identity, respectively) (data not shown). Regarding the diversity of the WDV isolates of the barley strain, we observed a relatively high variability (96.3–99.4%). Interestingly, however, the MP and CP also revealed a high level of amino acid sequence identity among barley strain isolates originating from different geographical regions (98.5–100%). Similar to wheat strain isolates, barley strain isolates showed greater variability also in the LIR and SIR.

Molecular characterization of Ukrainian and Hungarian WDV isolates was followed by phylogenetic analysis in order to compare their relationships with previously characterized wheat and barley isolates available from the GenBank database (Fig. 1). The phylogenetic analysis of WDV isolates showed that they were clearly distinguishable, both barley and wheat strains formed two clades. Isolates from Hungary, Germany, Czech Republic, Ukraine and Sweden clustered in clade 1. Interestingly, both Ukrainian isolates WDV-Uk-g and WDV-Uk-Miron showed closer relationship to WDV-HU-Pula and WDV-Swe-Enk2 isolates, respectively, than to each other. This is surprising as

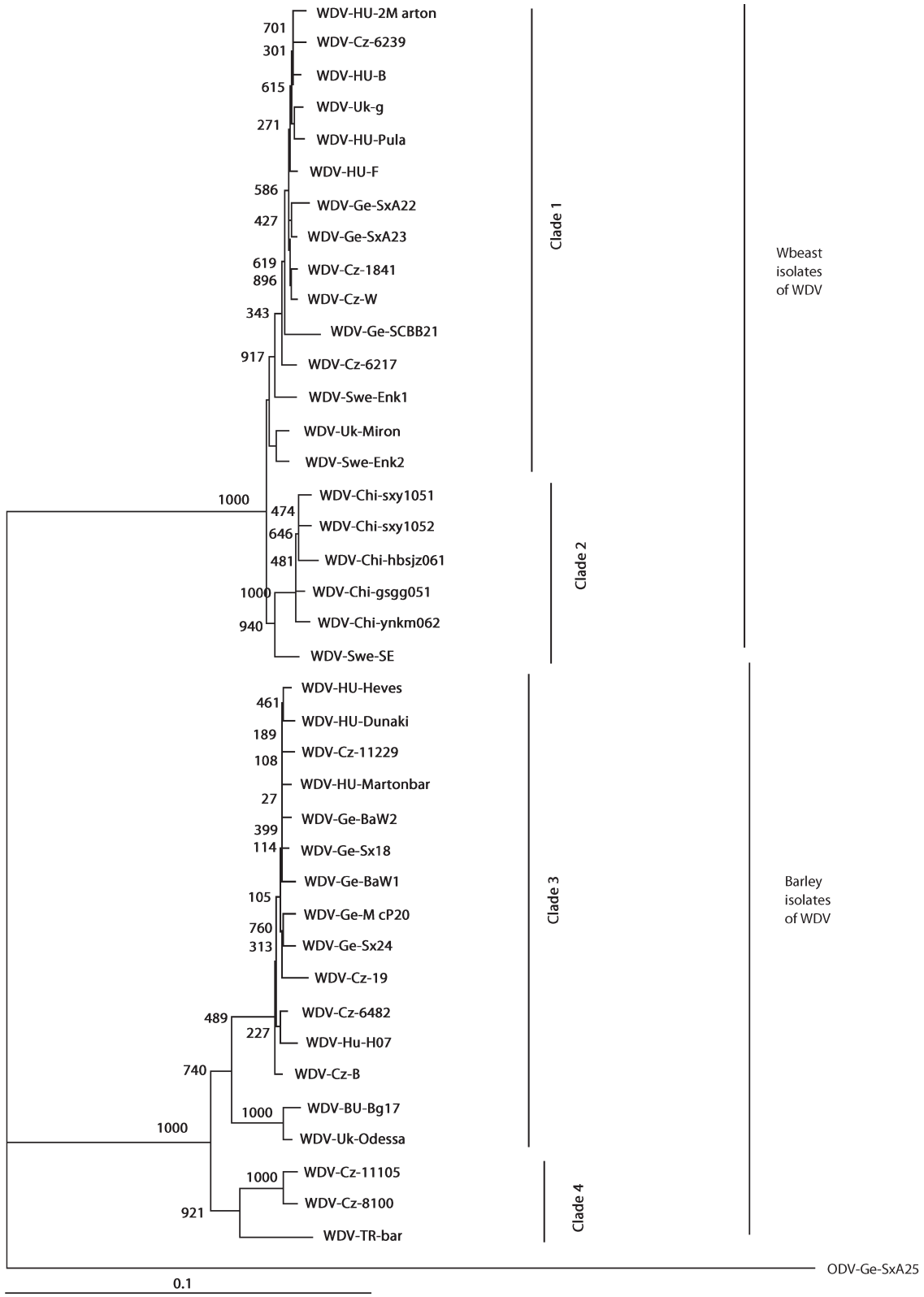

Fig. 1. Phylogenetic tree constructed by the UPGMA method for complete genome sequences of *Wheat dwarf virus* isolates. (Bootstrap values are indicated) The isolate ODV-Ge-SxA25 was used as the outgroup with a *ca.* 70% genome-wide nucleotide sequence identity with barley and wheat strain isolates of WDV.

according to the available literature data phylogenetic relationships of WDV isolates normally show high degree of dependence on the geographical origin of the virus (Köklü *et al.*, 2007). However, both WDV-Uk-g and WDV-Uk-Miron isolates came from the same geographical region of the Ukraine (Kiev region, central north of the Ukraine) and the sites of sampling were situated just approximately 100 km apart from each other. Apparently, other issues (such as vector occurrence and behaviour, plant cultivars cultivated at a given territory, agricultural techniques and, primarily, the initial virus source in the country) should be considered as well when evaluating spread and evolutionary divergence of WDV.

Clade 2 could be divided into two subgroups, one with wheat isolates from China and the other containing only the WDV-Swe-SE isolate from Sweden.

As for the isolates of barley strain of WDV, clade 3 could be divided into two subgroups, one with a divergent pool of isolates from Hungary, Germany and Czech Republic. The other subgroup comprised Ukrainian and Bulgarian isolates of WDV-BU-Bg17 and WDV-Uk-Odessa. In Clade 4, WDV-TR-bar isolate formed one subgroup and WDV-Cz-11105 and WDV-Cz-8100 formed the other one. These observations are in a good agreement with previous results (Schubert *et al.*, 2007, and Kundu *et al.*, 2009).

In conclusion, the results presented in this work have shown that Hungarian and Ukrainian isolates of WDV were divided into two distinct groups of wheat and barley strains. WDV-Uk-Odessa is the first barley strain isolate originating from wheat infected under natural conditions. At this point it should be mentioned that we have managed to identify the barley strain of WDV in a single wheat plant only once during the intensive two-year strain-specific PCR-based screening of WDV isolates in naturally grown cereal crops in Hungary and Ukraine. Hence the proven fact of WDV barley strain transmission to wheat plants by *P. alienus* is obviously an uncommon and rare event. Seemingly it may happen under natural conditions but only occasionally and possibly when virus concentrations in host plants are high enough to allow host range extension by overcoming typical limitations on virus-plant relationships.

In our opinion, the issue of WDV transmission by its vector needs further characterization especially by employing molecular approaches to identify virus genes and/or gene products (and preferably their vector counterparts) responsible for the transmission of the virus and its efficiency.

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