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Biological, serological and molecular characterisation of *Raspberry bushy dwarf virus* from grapevine and its detection in the nematode *Longidorus juvenilis*

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Abstract In 2003, *Raspberry bushy dwarf virus* was found for the first time in grapevine. Since this was the first report of a non-*Rubus* natural host, information about it is sparse. During this study the grapevine isolates were characterised biologically, serologically and genetically and compared with known information about *Rubus* isolates. Infected plant material was used for mechanical inoculation of test plants, and for serological characterisation with monoclonal antibodies. Most of RNA 2 was sequenced and compared with other sequences from the database. Grapevine isolates infected *Chenopodium murale* systemically, which is not known for raspberry isolates. They were differentiated from Slovenian raspberry isolates with three monoclonal antibodies using TAS-ELISA. Phylogenetic analyses clustered grapevine isolates separately from raspberry isolates. Additionally, the virus was detected using nested RT-PCR in *Longidorus juvenilis* nematodes collected in an infected vineyard. Grapevine isolates of RBDV are distinct from raspberry isolates.

Keywords Detection · ELISA · IC RT-PCR · Sequence · *Vitis vinifera*

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Introduction

Raspberry bushy dwarf virus (RBDV) is known to infect *Rubus* species worldwide. Many infected *Rubus* species and cultivars do not show any symptoms. In sensitive *Rubus* species and cultivars it induces yellows disease. It can cause premature defoliation, decreased vigour, leaf curling, necrosis, abortion of drupelets, death of lateral shoots and increased winter kill. It is also involved in inducing the disease known as 'bushy dwarf' or 'symptomless decline' in Lloyd George red raspberry (*Rubus idaeus*) when present together with aphid-borne *Black raspberry necrosis virus*. It is naturally transmitted by pollen to progeny and pollinated plants. It can be transmitted mechanically to and between different herbaceous hosts. RBDV has a bipartite genome with the bicistronic RNA 2 producing the movement protein (MP) and a subgenomic RNA that codes for the coat protein (CP) (Jones and Mayo 1998; Jones 2000).

RBDV is restricted mostly to *Rubus* species but it has been found to infect other plants after mechanical or graft inoculation. Jones et al. (1982) have described development of symptoms on *Cydonia oblonga* and *Pyronia veitchii* after graft-inoculation using scions infected with a type strain of RBDV. It can be grafted into *Fragaria vesca* and *Prunus mahaleb*, both without symptoms (Jones and Mayo 1998).

In 2003, we reported the infection of grapevines with RBDV in Slovenia, which was the first report of

this virus naturally infecting a host outside the genus *Rubus* (Mavrič et al. 2003). The virus was detected in symptomatic plants by DAS-ELISA and IC RT-PCR, and part of the CP was sequenced. Information about RBDV in grapevine is still sparse. Between 2003 and 2005, grapevine samples from all winegrowing regions of Slovenia were tested for RBDV infection. It was found in all winegrowing regions in seven white grapevine cultivars, Beli Pinot (=White Pinot) Chardonnay, Laški Rizling (=Italian Riesling), Malvazija (=Istrian Malvasia), Renski Rizling (=White Riesling), Sauvignon and Šipon (=Furmit). None of the samples of red cultivars tested was found to be infected (Virščck Marn and Mavrič 2006). Grafting experiments in 2003 and 2004 demonstrated the irregular distribution of RBDV in mother plants and grafted grapevines (Mavrič and Virščck Marn 2006).

Here we present further characterisation of RBDV isolates from grapevine. The isolates were analysed biologically, serologically and genetically and their characteristics compared with those of *Rubus* isolates. The host range following mechanical inoculation of test plants was determined. Serological reactions of grapevine and red raspberry isolates were compared in TAS-ELISA using three monoclonal antibodies. Most of RNA 2 from three grapevine and one raspberry isolate was sequenced and compared with sequences available in the Genbank database. Additionally, the virus was detected in nematodes from one of the vineyards with RBDV-infected vines.

Materials and methods

Plant material

In 2003 to 2006, 1,157 grapevine samples from different winegrowing regions of Slovenia were analysed for the presence of RBDV by DAS-ELISA. Six samples of *Vitis rupestris* were also included in the study. For comparison, RBDV infected red raspberry cv. Golden Bliss was included in the study. In 2002, grapevine grafts of cv. Laški Rizling were collected in the nursery, kept in the cold over the winter and planted in the greenhouse or outside in the following year. These plants were used for further serological and molecular characterisation. Plants of cv. Laški Rizling grown in the greenhouse were sampled to obtain information about virus distribution in the plants. Infected plants grown in the greenhouse showed no symptoms. Seeds were collected from plants of cv. Laški Rizling infected with RBDV. They were kept at 4°C for approximately 6 months, and then grown in a greenhouse. After about 3 months 390 seedlings were tested for RBDV infection with DAS-ELISA.

Mechanical inoculation of test plants

Chenopodium murale, *C. quinoa*, *Datura stramonium, Nicotiana benthamiana*, *N. clevelandii* and *N. glutinosa* were mechanically inoculated with sap from infected grapevine, using 0.02 M phosphate buffer containing 2% PVP (pH 7.4). Plants were grown in a growth chamber at 20–21°C with a 14 h photoperiod and were inspected regularly for symptom development; 14 to 21 days after inoculation they were tested by DAS-ELISA and/or IC RT-PCR for detection of symptomless infections.

ELISA

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using polyclonal antiserum against RBDV (Loewe Biochemica), was used to detect the virus in plant samples, to detect symptomless infections, and to confirm reactions on inoculated test plants. DAS-ELISA was performed according to manufacturer's instructions. Three monoclonal antibodies (D1, R2, R5) (R.R. Martin, USDA-ARS, Corvallis, OR, USA) were used in TAS-ELISA for serological characterisation. ELISA microtiter plates (Nunc, Denmark) were coated with RBDV IgG (Loewe Biochemica) as in DAS-ELISA. Plates were incubated overnight at 4°C and washed with ELISA wash buffer (PBS containing 0.05%Tween 20). Samples, diluted 1/20 with DAS-ELISA extraction buffer were incubated on plates overnight at 4°C and washed with ELISA wash buffer. Plates were incubated with DAS-ELISA extraction buffer supplemented with skimmed milk (20 gl^{-1}) for 30 min at 37°C, washed with ELISA wash buffer, incubated with monoclonal antibodies diluted in PBS (1/200) for 2 h at 37°C, washed with ELISA wash buffer and incubated with goat anti-mouse IgG-alkaline phosphatase (AP) conjugate (Sigma-Aldrich) diluted in DAS-ELISA extraction buffer (1/30.000) for 2 h at 37°C. After washing with ELISA wash buffer, the AP

substrate (Fluka) in the substrate buffer (9.7% diethanolamine, pH 9.8) was added to the plate and absorbance at 405 nm was measured after 1, 2 and 3 h.

Immuno-capture RT-PCR

Immuno-capture (IC) RT-PCR, using primers U1 and L4 (Kokko et al. 1996), was used to confirm some ELISA results and to detect the virus in inoculated test plants. For sequencing, two primer sets were used in IC RT-PCR, such that the amplicons represented most of RNA 2. Primer pair CPUP (CGGTACTGGTGAGGTG TATTT) and RNA12 (GGGGTTTGCTCAGCAAAC) amplified the CP region. Primer pair MPUP (CTGGA CATCTCGAGTTTGC) and MPLO (CGATTGGTG GAACAGCT) amplified the MP region. The amplification products were 1,328 and 1,072 bp, respectively.

Two hundred µl tubes were coated with RBDV antiserum (Loewe Biochemica) diluted 1/200 in ELISA coating buffer. Tubes were incubated overnight at 4°C and washed with ELISA wash buffer and distilled water. After washing, 25 µl of a reaction mix containing 50 pmol of primer RNA12, 5 µl 5 x M-MLV RT buffer (Promega), 5 µl dNTP mix (10 mM; Promega), 200 U M-MLV reverse transcriptase (M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant, Promega) and 20 U RNasin (Promega) were added to each tube. The reactions were incubated for 1 h at 50°C.

For the amplification, 48.5 µl of reaction mix consisting of 75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 µmol of each of the primers and 2.5 U Taq DNA Polymerase (Fermentas) were added to each tube containing 1.5 µl cDNA mix. The amplification consisted of initial denaturation of 5 min at 94°C, followed by 40 cycles of amplification with a thermal profile of 1 min at 95°C, 1.5 min at 55°C and 1.5 min at 72°C and final elongation for 10 min at 72°C. Amplified products were analysed on 1% agarose gels and stained with ethidium bromide.

Sequencing and sequence analysis

The selected amplification products of 1,328 and 1,072 bp were purified and cloned into pGEM-T easy vector (Promega) according to the manufacturer's instructions. Transformed colonies were selected by blue/white screening and subsequent PCR. Plasmids were isolated from selected colonies and sequenced (Macrogen). The nucleotide sequences of the amplification products and their deduced amino acid sequences were compared with sequences in the GenBank. They were deposited in the GenBank under accession numbers EU796085, EU796086, EU796087, EU796088, EU796089 and EU796090.

Sequence analyses were conducted using tools available on the Internet (http://www.ncbi.nlm.nih.gov/BLAST/) and BioEdit version 7.0.5.3 (Hall 1999). Multiple alignments were generated using the Clustal X programme (Thompson et al. 1997) with default parameters. Phylogenetic analyses were conducted using the MEGA 3 programme (Kumar et al. 2004). Phylogenetic trees were constructed using the neighbour-joining method and bootstrap analysis with 1,000 repetitions.

Nucleotide sequences and deduced movement protein (MP) amino acid sequences were compared with published sequences S55890 from black raspberry and DQ120126 from *Rubus multibracteatus*. Deduced coat protein amino acid sequences were compared with published sequences S55890 from black raspberry, DQ120126 from *R. multibracteatus*, AF259798 strain Can-S from Canby red raspberry after several passages in *N. benthamiana*, AF259795 strain D200 the type strain from Lloyd George red raspberry, AF259796 strain D-1, obtained from strain D200 after several years of passaging in *C. quinoa* and D01052 strain R-15 from Malling Jewel red raspberry.

Detection of RBDV in nematodes using nested RT-PCR

The nematodes used for RBDV detection tests were extracted by the whirling-motion method (Hržič 1973) from fresh soil samples, from samples stored at 4°C for 4 months and from samples stored at 4°C for 8 months. In total, nine samples containing between three and 44 individuals of *Longidorus juvenilis* were tested for RBDV. Nematodes of mixed developmental stages or adult females were transferred into 2 µl of distilled water and stored at −80°C until analysed. Total RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen) as described by Demangeat et al. (2004).

Nested RT-PCR using RBDV specific primers U1 (5′-GCTGTTCCACCAATCGTTA-3′), L3 (5′-

CGTCGACGGCACCGCCCACCACA-3′) and L4 (5′-GCTATGCCGTTTATCTCAC-3′) described by Kokko et al. (1996) was used to detect RBDV in extracts of total RNA from nematodes. For cDNA synthesis, 10 µl of extracted total RNA were added to 15 µl of reaction mix containing 50 pmol of primer L4, 5 µl ×5 M-MLV RT buffer (Promega), 5 µl dNTP mix (10 mM) (Promega), 200 U M-MLV reverse transcriptase (M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant, Promega) and 20 U RNasin (Promega). The reactions were incubated for 1 h at 50°C.

Ten µl of the RT reaction were added to 40 µl of reaction mix consisting of 75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 µmol of each of the primers U1 and L4, and 2.5 U Taq DNA Polymerase (Fermentas) for the first PCR. The amplification process consisted of initial denaturation of 5 min at 94°C, followed by 40 cycles of amplification with thermal profile of 1 min at 95°C, 1.5 min at 55°C and 1.5 min at 72°C and final elongation for 10 min at 72°C. One µl of the amplification product was used in a second amplification which was carried out as described above, except that primers U1 and L3 were used. Amplified products were analysed on 1% agarose gels and stained with ethidium bromide.

Results

Plant material

Out of the 1,157 grapevine samples collected in 2003–2006 from all three winegrowing regions of Slovenia, 334 were infected with RBDV. Some samples were also infected with *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine leafroll-associated viruses 1* and *3* (GLRaV-1 and GLRaV-3). The results of years 2003–2005 have been presented by Viršček Marn and Mavrič (2006).

In 2006, 161 samples were analysed and 99 shown to be RBDV positive by DAS-ELISA. In 2006, eight out of 20 samples of the red grapevine cv. Modri Pinot (=Pinot Noir) from Podravje region were found to be positive by DAS-ELISA. An additional sample with a threshold DAS-ELISA result was confirmed to be positive by IC RT-PCR. These were the first infections with RBDV found in a red cultivar. In previous years none of the 314 samples of different red cultivars was found to be infected (Viršček Marn and Mavrič 2006). None of the six *Vitis rupestris* samples was found to be infected with RBDV. Other cultivars in which infection with RBDV was found are Beli Pinot, Chardonnay, Laški Rizling, Malvazija, Radgonska ranina, Renski Rizling, Rizvanec, Sauvignon, Šipon and Traminec (Viršček Marn and Mavrič 2006 and this study).

One infected and one non-infected greenhouse grown plant of cv. Laški Rizling were sampled to obtain information on virus distribution. Old, medium and young leaves, shoot tips, petioles, inner bark of stems, roots and berries were sampled. The virus was detected in all organs of the infected plant, though not reliably in the leaves and roots but was always detected in the other plant parts. It could also be detected in dormant buds on material stored at 4°C.

All 390 tested seedlings from the seeds collected from the infected plants of cv. Laški Rizling were negative by DAS-ELISA. This is a strong indication that RBDV is not transmitted by seeds in grapevine cv. Laški Rizling.

Serological differentiation and test plant reactions

Three monoclonal antibodies (D1, R2 and R5) were used to differentiate between raspberry and grapevine isolates in TAS-ELISA. An isolate from infected red raspberry cv. Golden Bliss reacted with all three monoclonal antibodies. Grapevine isolates reacted only with R2 and R5 but not with D1.

Different inoculation buffers were tested for inoculation and found to be equally effective. The inoculations were successful only with young plant material collected in spring. Local and systemic necrotic lesions developed on *C. murale*. Local chlorotic spots were observed on *C. quinoa*. No symptoms were observed on other inoculated plants. RT-PCR results confirmed systemic infection of *C. murale*.

Sequencing and sequence analyses

Most of RNA 2 of one raspberry isolate, three grapevine isolates, one raspberry isolate on *Chenopodium murale* and one grapevine isolate on *C. murale* were sequenced during this study. Sequences were deposited in the GeneBank under accession numbers EU796088 (red raspberry, isolate RR-1), EU796087

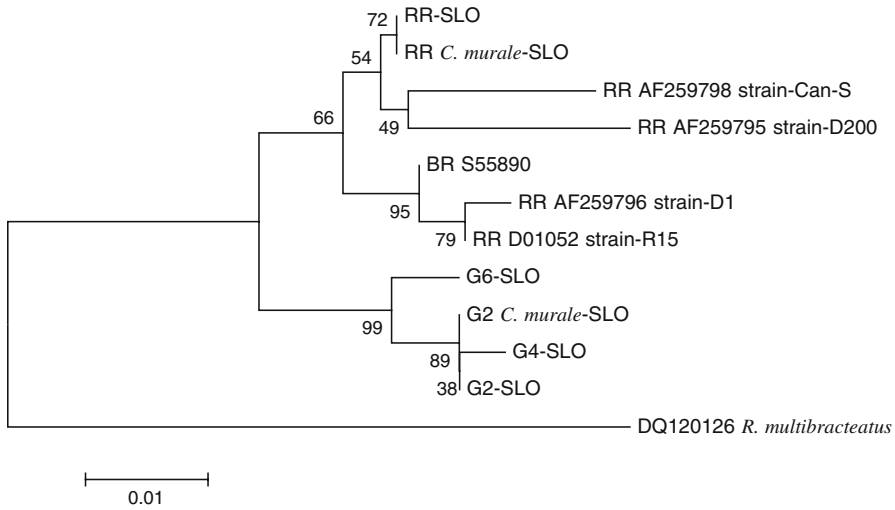

Fig. 1 Phylogenetic tree constructed on the basis of coat protein amino acid sequences. Multiple alignments were made using Clustal X, and analysed by the neighbour-joining method, with 1,000 bootstrap replications. *RR* red raspberry, *BR* black raspberry, *G* grapevine, *SLO* sequences of Slovenian isolates

(grapevine 2, isolate GR-2), EU796086 (grapevine 4, isolate GR-4), EU796085 (grapevine 6, isolate GR-6), EU796089 (*C. murale*—raspberry, isolate CmRR-1) and EU796090 (*C. murale*—grapevine 2, isolate CmGR-2). The CP and MP sequences of the *C. murale* isolate from grapevine was identical to the original grapevine isolate.

Phylogenetic analyses made with deduced amino acid sequences of CP (Fig. 1) and MP (Fig. 2) show three groups of isolates, group 1 contained the red and black raspberry isolates, group 2 the grapevine isolates and group 3 the isolate from *R. multibracteatus*.

Detection of RBDV in nematodes

Nematodes were extracted from five fresh soil samples and from four samples stored at 4°C for several months. The nematodes were tested for RBDV by nested RT-PCR. Nematodes from four fresh soil samples tested positive for RBDV and nematodes from one fresh soil sample tested negative. Two soil samples were stored in a refrigerator for 4 months, after which nematodes from both tested positive for RBDV. These PCR products were also sequenced, confirming the nested RT-PCR results. Two soil samples were stored in a refrigerator for 8 months and the extracted nematodes tested positive for RBDV in one of them (Fig. 3).

Discussion

RBDV was found for the first time infecting grapevine, the only known non-*Rubus* natural host, in 2003 in Slovenia (Mavrič et al. 2003). It was identified in grapevine grafts of cv. Laški Rizling with curved line patterns and yellowing of the leaves (Fig. 4). The aim of this research was to obtain additional information about RBDV isolates from

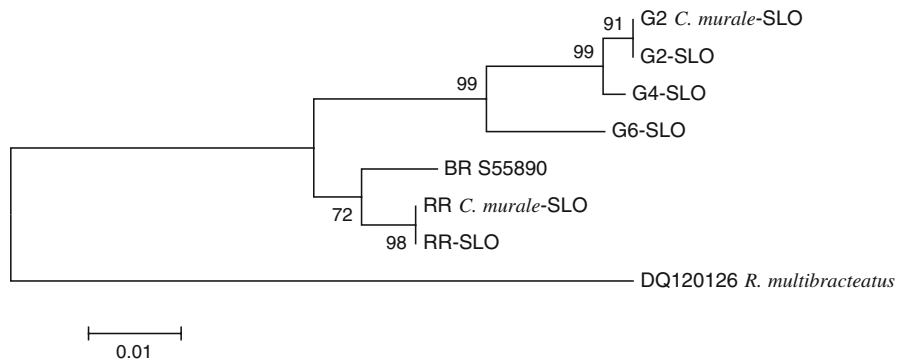

Fig. 2 Phylogenetic tree constructed on the basis of the movement protein amino acid sequences. Multiple alignments were made using Clustal X, and analysed by the neighbour-joining method, with 1,000 bootstrap replications. *RR* red raspberry, *BR* black raspberry, *G* grapevine, *SLO* sequences of Slovenian isolates

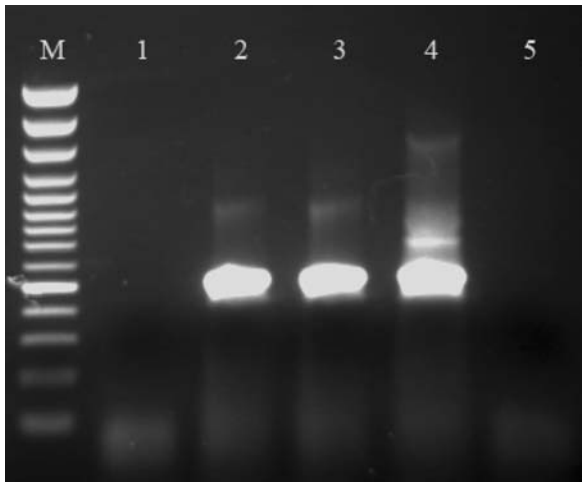

Fig. 3 Agarose gel electrophoresis of nested RT-PCR products amplified from total RNA samples from the nematodes extracted from the soil around infected plants. *M* DNA size marker 100 bp (Fermentas), *1 X. wuittenezi*, five individuals, soil sample stored for 8 months, *2 L. juvenilis*, six individuals, soil sample stored for 8 months, *3 L. juvenilis*, 44 individuals, soil sample stored for 4 months, *4* positive control (cDNA from RBDV infected raspberry), *5* water control

grapevine and to compare them with common raspberry isolates.

RBDV was also detected in several grapevine cultivars with less distinct symptoms (Virščck Marn and Mavrič 2006). It was found in cv. Laški Rizling in all winegrowing regions of Slovenia (Podravje, Posavje and Primorje). RBDV was most widespread in the Podravje winegrowing region, where this cultivar is widely grown. In the Primorje region it was found mainly in the Vipava valley, where cv. Laški Rizling is grown; in other locations of this region this cultivar is not very popular. RBDV was also found in many white grapevine cultivars (Beli Pinot, Chardonnay, Malvazija, Radgonska ranina, Renski Rizling, Rizvanec, Sauvignon, Šipon, Traminec) and in 2006 also in the red cv. Modri Pinot. This was the first red cultivar found to be infected. The reason for this could be the fact that red cultivars are mostly grown in Primorje and Posavje, where the incidence of the virus is lower.

Results from the previous (Mavrič and Virščck Marn 2006) and the present study also show that RBDV is irregularly distributed in individual infected plants. This information is important for its reliable detection in grapevine. When leaves are used for RBDV detection, several leaves from different parts of the plant should be sampled.

In raspberry, RBDV is seed transmitted. Up to 77% of seedlings from red raspberry were infected when both parents were infected (Jones et al. 1996). Virus was found to be seed transmitted also in *R. phoenicolasius* (up to 15%) and *Fragaria vesca* (1–2%) (Jones and Mayo 1998). No seed transmission was found in *C. quinoa*. Our results show that RBDV is not seed transmitted or transmitted at a low efficiency in grapevine cv. Laški Rizling.

After mechanical inoculation of test plants, local and systemic symptoms were observed on *C. murale* with grapevine isolates. Plants also tested positive for RBDV by DAS-ELISA. Systemic reaction of *C. murale* to RBDV infection has not been reported previously. Raspberry reactions usually include only local lesions on *C. murale* and a systemic reaction on *C. quinoa* (Jones and Mayo 1998). Raspberry isolates also infect *Nicotiana clevelandii*, but without symptoms, whereas the grapevine isolates did not infect this plant species. The reactions we observed with grapevine isolates were clearly different from those described for raspberry isolates.

Several monoclonal antibodies (MAbs) against RBDV were produced by Martin (1984). Several RBDV isolates were tested with these MAbs in DAS-ELISA and they all reacted with all MAbs (Jones et al. 1996). Chamberlain et al. (2003) described a strain of RBDV from *Rubus multibracteatus* (RBDV-China) which could be clearly differentiated using three of the aforementioned MAbs in TAS-ELISA. MAb D1 did not detect RBDV-China, whereas MAbs R2 and R5 did. We tested the same MAbs in TAS-ELISA

Fig. 4 Typical symptoms observed on RBDV-infected grapevine plants of cv. Laški Rizling

with our grapevine and red raspberry isolates. Red raspberry isolates reacted strongly with all three MAbs, whereas grapevine isolates reacted only with MAbs R2 and R5. To our knowledge this is the second group of isolates which could be differentiated with these MAbs.

Phylogenetic analysis clearly showed three clusters of isolates with all sequences used (whole available nucleotide sequences, CP and MP amino acid sequences). Isolates from red and black raspberries formed one cluster, grapevine isolates another and the isolate from *R. multibracteatus* was separate from all the others.

In addition, RBDV was detected, using total RNA isolation followed by nested RT-PCR, in *L. juvenilis* nematodes from soil collected at the same location as RBDV-infected plants. Specific amplification products were found in nematodes soon after they were collected in the field, as well as after 4 and 8 months of storage of infested soil in a refrigerator in our laboratory. To our knowledge this is the first detection of RBDV in nematodes. It is known that some viruses can be detected and remain infective in their vectors during long periods of storage. *Xiphinema index* was still infective after up to 8 months of storage in moist soil (Taylor and Brown 1997). GFLV could be detected by RT-PCR in *X. index* extracted from soil samples kept at 7°C and 20°C for up to 4 years of storage (Demangeat et al. 2005). *Xiphinema americanum* transmitted TRSV after 9 months of storage at 8°C (Taylor and Brown 1997) and *Xiphinema rivesi* transmitted ToRSV to bait plants after 2 years of storage in natural soil (Bitterlin and Gonsalves 1987). But viruses can also be detected in nematodes, which are not their vectors. ArMV and SLRV were detected in *Longidorus elongatus* by the 'slash test' method, although they are not transmitted by it (Taylor and Brown 1997). The possible role of *L. juvenilis* in RBDV transmission is still under investigation in our laboratory.

In recent years, two distinct isolates of RBDV were identified from new hosts, one from grapevine (Mavrič et al. 2003) and one from *R. multibracteatus* (Chamberlain et al. 2003). They are both serologically different from known raspberry isolates. Sequence identities and phylogenetic analyses separate them from raspberry isolates. Another *Idaeovirus* isolate was found on *Citrus* sp. (Derrick et al. 2006). The partial sequence of RNA 2 of the citrus-infecting virus is considerably different from other sequences, including the grapevine and *R. multibracteatus* sequences. All these new findings suggest that the *Idaeovirus* genus of plant viruses has a wider host range than previously thought. It should receive more attention in the near future.

Grapevine-infecting RBDV isolates are widespread in Slovenia. RBDV is present in all winegrowing regions of the country and was found in many grapevine cultivars. More research on the epidemiology and economic importance of RBDV in grapevine needs to be carried out to determine the threat it poses to grapevine production and fruit quality and the extent of its distribution in other parts of the world.

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