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# Simultaneous detection and genetic variability of stone fruit viroids in the Czech Republic

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**Abstract** In this paper, we report a large-scale survey for the incidence of *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) in stone fruit collections and commercial orchards in the Czech Republic. From the 645 samples analysed, PLMVd was detected in 80 (26.6%) of peaches and the HSVd in 3 (1.3%) of apricot and 1 (0.33%) of peach trees. Sixty-seven accession of peach (44.6%) from the Czech Clonal GeneBank were infected by PLMVd. In addition, we used naturally infected trees to standardise the simultaneous detection of PLMVd and HSVd plus host mRNA as the control by means of one-step multiplex RTC-PCR. Eleven PLMVd and two HSVd isolates were sequenced and analysed. All the PLMVd variants were highly homologous (97–100%) to previously reported PLMVd variants from Tunisian peach and almond trees, and clustered together in the previously reported phylogenetic group III. The HSVd variants obtained from apricot and peach trees were included in the previously proposed recombinant group PH/cit3.

**Keywords** *Prunus* · PLMVd · HSVd · Multiplex RT-PCR · Stone fruit viroids

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Viroids are small, single-stranded, circular, pathogenic RNAs that do not code for proteins. They induce diseases in plants by direct interaction with host factors through a mechanism yet unidentified (Tabler and Tsagris 2004). Viroids should be considered as highly gifted, modern RNA parasites that have eliminated all the dispensable 'extravagance' of viral pathogens to make use of the host in the most sophisticated manner (Tabler and Tsagris 2004). The impact of viroids infecting woody perennial fruit trees is conditioned by the long life of the fruit trees which favours continuous infection events. Moreover, in this type of crop two sources of contamination are possible, the rootstocks and the varieties, which also increase the frequency of multiple infections. Two viroids are so far known infecting stone fruit trees,

*Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). PLMVd is a serious peach pathogen (Flores et al. 2003) listed as a quarantine pathogenic agent by the European Plant Protection Organization. HSVd was found to be associated with disorders of serious economic importance e.g. dapple fruit in plum and peach (Shikata 1990). Recently, a new important apricot disease, known as 'degeneration', has been associated with HSVd in Spain (Amari et al. 2007).

Once fruit trees become infected with viroids there is no cure system yet available. Most of procedures that can be used effectively involve measures designed to reduce sources of infection inside and outside the crop. Development of strategies to control and/or eliminate diseases caused by viroids, requires knowledge on variation and evolution of viroid populations coupled with early detection, followed by eradication of infected sources.

Early detection by means of sensitive diagnostic methods is the main way to control viroid diseases. Molecular techniques (polymerase chain reaction (PCR) and nucleic acid hybridisation) are considered reliable, fast and sensitive detection techniques compared with biological methods, and could be applied to the large-scale and multiple detection of plant pathogens. Procedures that allow the simultaneous detection and/or identification of different plant pathogens are desirable for routine diagnosis because they require less time, labour and cost. In this context, the simultaneous detection by reverse transcriptase-PCR (RT-PCR) has been used successfully for routine diagnosis of plant pathogens (Bertolini et al. 2001; James et al. 2006). In addition, to ensure the safe interpretation of negative results, specific primers to host mRNA were included as internal controls in the RT-PCR reaction to detect simultaneously pome and stone fruit viruses (Menzel et al. 2002; Sanchez-Navarro et al. 2005; Hassan et al. 2006).

Here, we report a large-scale survey for the incidence of PLMVd and HSVd in several stone fruit collections and commercial orchards in the Czech Republic and use naturally infected trees to standardise the simultaneous detection of both viroids by means of one-step RT-PCR. In addition, we characterise the sequence variants of HSVd and PLMVd isolated from the infected cultivars. A total of 645 samples were collected from different cultivar collections and orchards of stone fruit trees in the Czech Republic. Except for some apricot and peach trees with wilting and leaf roll (symptoms characteristic for phytoplasma infection), no visible symptoms were observed during the collection of the samples to be evaluated. Samples were mainly from peach and apricot from the Czech Clonal Genebank and nuclear stocks (pre-basic) located in Lednice (South Moravia region). The *Prunus* spp. accessions are from Czech origin and others were imported from the United States and Italy (Table 1).

To complete the survey, mother trees from the main commercial nursery at Valtice and private commercial orchards, which now prevail in the trade and services, were sampled in this study. The collections were made from European plum (*P. domestica*), peach (*P. persica*), apricot (*P. armeniaca*), sweet cherry (*P. avium*), sour cherry (*P. cerasus*), myrobalan (*P. cerasifera*), blackthorn (*P. spinosa*), and wild peach (*Persica vulgaris*). Leaves from different parts of the canopy were taken during spring and summer. The samples were stored in —80°C until processed and subsequently analysed for the presence of HSVd and PLMVd using dot-blot hybridisation as previously described (Más and Pallás 1995). The nucleic acid extraction was performed from leaf tissue (0.2 g) as previously described (Astruc et al. 1996) and resuspended in 50 μl of sterile water. Aliquots (5 μl) were spotted on a nylon membrane, hybridised over night at 70°C with full-length digoxigenin-labelled PLMVd and HSVd riboprobe, and detected as recommended by the supplier (Roche, DIG Luminescent Detection Kit for Nucleic Acid).

From the 645 samples analysed (300 peach, 50 plum, 220 apricot, 50 cherry, and 25 wild *Prunus* spp.), 80 peaches were positive for PLMVd and three apricots for HSVd (Table 2). No apricots, cherries, plum or wild *Prunus* spp. trees were infected by PLMVd. Sixty-seven accessions of peach (44.6%) from the Czech Clonal GeneBank were infected by PLMVd. In nuclear stocks, four peach samples of pre-basic materials were found to be infected by PLMVd; it was interesting to observe that all infected plants in this collection belonged to cv. Frederica imported from Italy. Table 3 shows that the PLMVd incidence in peach was 26.6% of the total, confirming that peach is the favourite host of PLMVd (Flores et al. 2003). This result fits well with data reported from other countries (Badenes and Llácer 1998; Michelutti et al. 2004). However, HSVd was detected in three out of a total of 220 apricot trees, plus

**Table 1** List of sequence PLMVd and HSVd isolates

| Viroid | Host | Cultivars | Variants | Length (nt) | Location | Origin |
|---|---|---|---|---|---|---|
| PLMVd | Peach | F. Morettini | FM1 | 339 | Prague | Italy |
| | | | FM2 | 339 | | |
| PLMVd | Peach | Federica | Fr1 | 338 | Lednice | Italy |
| | | | Fr12 | 338 | | |
| | | | Fr2 | 337 | Lednice | Italy |
| | | | Fr21 | 337 | | |
| | | | Fr3 | 337 | Lednice | Italy |
| | | | Fr31 | 338 | | |
| PLMVd | Peach | Envoy | Ev34 | 339 | Lednice | USA |
| | | | Ev35 | 337 | | |
| PLMVd | Peach | Daniela | Dn27 | 339 | Lednice | Italy |
| | | | Dn28 | 339 | | |
| PLMVd | Peach hybrid | Barier | Br3 | 339 | Lednice | Italy |
| | | | Br5 | 339 | | |
| PLMVd | Peach | Fairhaven | Fh12 | 338 | Valtice | USA |
| | | | Fh13 | 339 | | |
| PLMVd | Peach | Dixired | Dx1 | 339 | Valtice | USA |
| | | | Dx2 | 340 | | |
| PLMVd | Peach | Cresthaven | Cr6 | 338 | Tuřany | USA |
| | | | Cr7 | 338 | | |
| PLMVd | Peach | Redwin | Rd22 | 338 | Tuřany | USA |
| | | | Rd23 | 339 | | |
| HSVd | Apricot | M-Le-1 | M-Le-1 | 297 | Lednice | Czech |
| HSVd | Peach | B-VA-2 | B-VA-2 | 297 | Lednice | Czech |

one accession of peach rootstocks, B-VA-2 (Table 2). Generally, the incidence of HSVd in the tested samples seemed to be low and consistent with results reported previously (Mandic et al. 2008).

Representative infected trees were used to optimise the simultaneous detection of HSVd and PLMVd by one-step RT-PCR. Total RNA was extracted from 100 mg of plant tissues using the Qiagen RNeasy total RNA kit (QIAGEN, mbH, Hilden, Germany) and eluted in 80 µl of sterile water. The Qiagen®One-step RT-PCR kit (QIAGEN) was selected for single and multiplex RT-PCR. For simultaneous RT-PCR detection, we used a cocktail of primers previously described for the specific amplification of PLMVd (Loreti et al. 1999) and HSVd (Astruc et al. 1996). A primer pair specific to mRNA of the mitochondrial *nad5* gene was used as the amplification internal control (Menzel et al. 2002).

**Table 2** PLMVd infection distributed by peach sample type and incidence level

| Type | Tested trees (n°) | Infected trees (n°) | Infection rate (%) |
|---|---|---|---|
| Orchard | 80 | 7 | 8.7 |
| Nursery | 20 | 2 | 10 |
| Nuclear stock | 50 | 4 | 8 |
| Germplasm | 150 | 67 | 44.6 |
| Total | 300 | 80 | 26.6 |

**Table 3** Results of molecular hybridisation for PLMVd and HSVd

| Species | No. of trees | | Viroid infection | | Infection rate (%) |
|---|---|---|---|---|---|
| | Tested | Infected | PLMVd | HSVd | |
| Peach | 300 | 81 | 80 | 1 | 27 |
| Apricot | 220 | 3 | – | 3 | 1.3 |
| Plum | 50 | – | – | – | – |
| Cherry | 50 | – | – | – | – |
| Other *Prunus* spp. | 25 | – | – | – | – |
| Total | 645 | 84 | 80 | 4 | 13 |

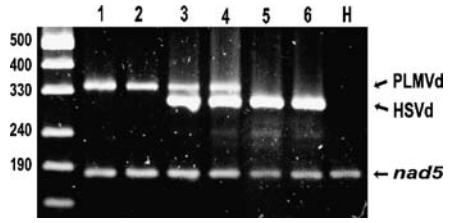


**Fig. 1** Simultaneous detection of PLMVd and HSVd by one-step multiplex RT-PCR primed by viroid-specific and *nad5* primers. RNA sources were two field peach trees with a single and double viroid infection. Lanes 1 and 2: PLMVd-infected samples; 5 and 6: HSVd-infected; lanes 3 and 4: double-infected; H: healthy GF-305

The most efficient amplification of all targets under the same RT-PCR conditions was obtained using a cocktail containing 0.7 μM of the PLMVd-specific primers and 0.5 μM of the HSVd-specific primers, plus 0.25 μM of the *nad5* primers as the amplification control. The cycling conditions were RT (30 min at 50°C), activation of the HotStart Taq polymerase (15 min at 95°C) followed by 35 cycles of 30 s at 94°C, 45 s at 60°C, 60 s at 72°C), and the final extension step (7 min at 72°C). Fig. 1 shows the viroid-specific amplification products plus the host mRNA either in peach trees naturally infected by PLMVd and/or HSVd. Three amplified products of expected sizes were observed, 339 bp for PLMVd; 300 bp for HSVd and 181 bp for host mRNA. No amplification was observed in healthy and negative controls. These results reinforce previous reports indicating that the simultaneous RT-PCR assay is a highly applicable diagnostic technique for stone fruit viroids. In addition, the co-amplification of host mRNA as in internal control increases the potential of the technique to avoid false negative results (Menzel et al. 2002; Navarro et al. 2004; Sanchez-Navarro et al. 2005; Hassan et al. 2006).

Amplified products were cloned in the pGEM-T vector (Promega, Madison, WI, USA) and sequenced. Multiple alignments of nucleotide were obtained using the default options of Clustal X 1.8. Phylogenetic analysis was done using the minimum evolution method of phylogenetic inference (Rzhetsky and Nei 1993) with 1000 bootstrap replicates. The version 2.1 of the Molecular Evolutionary Genetics Analysis software MEGA version 2.1 was utilised (Kumar et al. 2001).

In this study we analysed HSVd variants isolated from two Czech-native *Prunus* spp. Apricot (M-Le-1) and Peach (B-VA-2) (Table 1). The HSVd.B-VA-2 variant sequence (297 nt long) was 100% homologous to the previously described isolate *apr*18, a variant sequence identified in apricot plants (cv. *Boccuccia spinosa*) from Cyprus (Amari et al. 2001). The isolate HSVd.M-Le-1 (297 nt long) was found to be very similar to *apr18* ('U' 265 change to 'A') and Apricot Ring Pox (U 257 change to G) isolates (Ragozzino et al. 2004), suggesting that this new variant is composed of a mosaic of informative

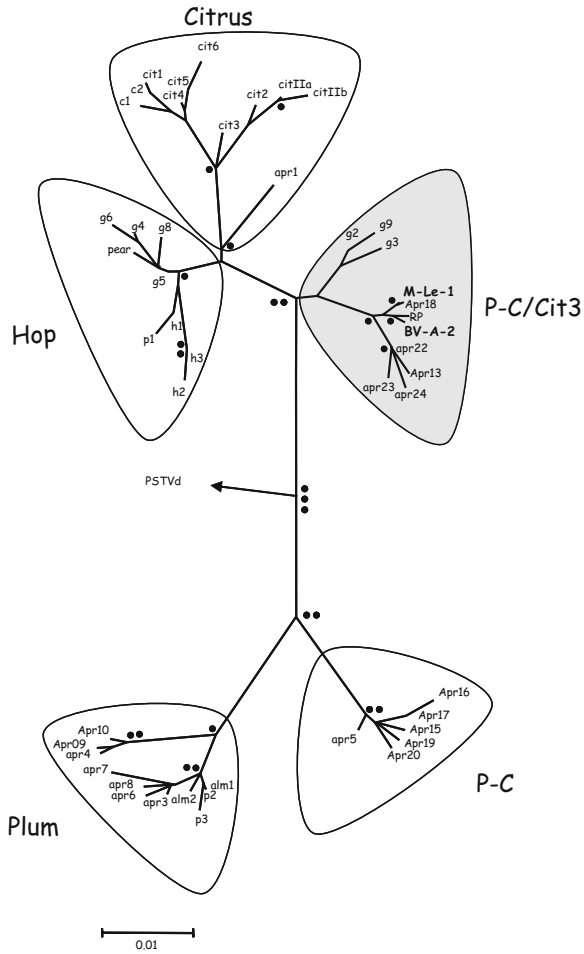


**Fig. 2** Phylogenetic tree of HSVd sequence variants. The five phylogenetic groups are delineated. The HSVd-Czech variants sequenced in this work M-L-1 and B-VA-2 are in bold letters. An arrow indicates the position of PSTVd, included in the analysis as an out-group. Dots near nodes indicate the statistical value of the node as determined by bootstrap analysis (1000 replicates). ●●●, Node detected in 100% of replicates; ●●, node detected in 80–100% of replicates; ●, node detected in > 50% of replicates

changes from both representative variants. Phylogenetic analysis (Fig. 2) shows that the HSVd.B-VA-2 and HSVd.M-Le-1 could be included in the previously identified recombinant group PH/cit 3 (Amari et al. 2001). It is worth noting that the changes detected in HSVd-Czech isolates were located at variable residues among HSVd sequences, in loops on the left of the rod-like molecule that included the pathogenicity (P) domain, reinforcing the hypothesis that HSVd variability is restricted to certain polymorphic positions (Amari et al. 2001)

PLMVd variants were isolated from eleven asymptomatic peach sources from the Czech Republic (Table 1). For each isolate two full-length cDNA clones were sequenced. All the PLMVd variants ranged in size from 336–340 nt being similar in size to previously described isolates (Ambrós et al. 1998; Pelchat et al. 2000; Malfitano et al. 2003).

The PLMVd variants described in this work were highly homologous (97–100%) to the previously reported PLMVd, isolated from Tunisian peach, pear and almond trees clustered in group II (subgroups IIA and IIB) by Hassen et al. (2007). These variant sequences are characterised by seven informative polymorphisms (U17, A24, A/U 42, A178, U 185, U/A 299 and A321), maintained in Czech PLMVd isolates, except for two variations (C17 and G24) in the DX2 isolate. Future studies will be performed to determine if the similarities in this informative polymorphism between Czech and Tunisian isolates have some biological and/or epidemiological significance.

PLMVd isolates have historically been classified in three main phylogenetic groups, named I, II and III (Ambrós et al. 1998). The comparison between the sequence of the isolates described here and those previously characterised, revealed that all obtained sequence variants could be included together in group III (Fig. 3). However, it is worthy to note that a high intra-group variability was observed in relation to the variant sequences previously clustered in this group, suggesting the possibility that this phylogenetic classification should be revised in futures studies.

In short, we have detected and identified new HSVd and PLMVd variants in stone fruit orchards in the Czech Republic. Further improvements for RT-PCR were achieved by optimisation of one-step multiplex RT-PCR enabling simultaneously the detection of PLMVd, HSVd and host mRNA as the internal control in one assay. Coupling the fast and simple RNA extraction method with one-step multiplex RT-PCR for the detection and identification of stone fruit viroids can help to minimise the time and labour required for the diagnosis of these viroids. This method is efficient, specific, and reduces the possibility of false-positives, since all steps are performed in the same tube avoiding cross-contamination. In addition, the use of the internal control minimises the risk of obtaining false-negative results, which is highly desirable for routine testing.

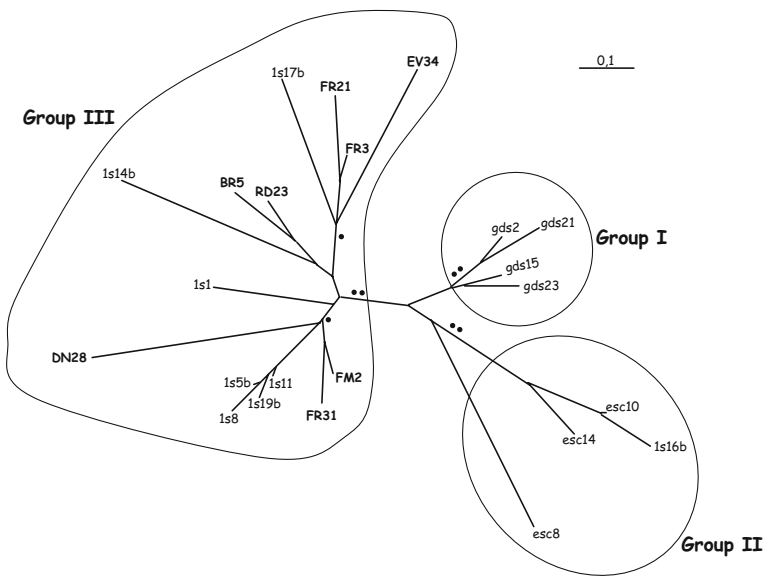


**Fig. 3** Phylogenetic tree of the PLMVd variants. The analysis was based on the distance calculated between representative eight sequence variants described in this work and 15 PLMVd sequences previously described. Three phylogenetic groups described by Ambrós et al. (1998) are shown. Dots indicate the statistical value of the node as determined by bootstrap analysis (1000 replicates). ●●: node detected in > 75% of replicates; ●: node detected in > 50% replicates

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