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FULL RESEARCH PAPER

Incidence and genetic diversity of Peach latent mosaic viroid and Hop stunt viroid in stone fruits in Serbia

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Abstract Tissue-imprint hybridization (TIH) assay was validated for large-scale detection of *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). All 72 collected leaves (100%) from 2 PLMVd- and 2 HSVd-infected trees were positive in TIH, regardless of the geographic orientation of the scaffold, level of the canopy and position of the leaf in the shoot. In a large-scale survey in Serbia, we tested by TIH 871 trees of stone fruits, representing 602 cultivars from fruit collections in Belgrade, Čačak and Novi Sad. PLMVd was detected in 185 (50%) peach trees or 95 (54%) cultivars and HSVd in 2 apricot trees and cultivars (2%). The occurrence of HSVd is a new report for Serbia. No viroid infection was found in European plums, sweet cherries, sour cherries and wild *Prunus* spp. PLMVd-infected peach cultivars originated from the world's main breeding centres of this crop. Western European and Asian cultivars were the most infected (58%) followed by those originating from North America (50%). Nine PLMVd and two HSVd isolates were sequenced and analyzed. All showed PMLVd sequences clustered together in the previously reported phylogenetic group III. Both HSVd isolates were found to be derived from recombinant events, but that of the cv. Saturn represented a putative new phylogenetic group of HSVd.

Keywords Prunus · PLMVd · HSVd · Tissue-imprint hybridization · RT-PCR

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Introduction

Viroids are small circular single-stranded RNA molecules with sizes ranging between 246 and 401 nucleotides (Diener 1991; Tabler and Tsagris 2004; Flores and Pallas 2006). They are the smallest known plant pathogens and cause several economically significant crop diseases. Viroid species are classified into two families, *Pospiviroidae* and *Avsunviroidae* (Flores et al. 1998). Viroids in the family *Pospiviroidae* contain a central conserved region (CCR) in their RNA molecule and replication takes place in the nucleus. On the other hand, members of the family *Avsunviroidae* lack CCR, replicate in the chloroplast and are able to self-cleave through hammerhead ribozymes (Flores and Pallas 2006). *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) are the viroids reported to affect

Prunus spp., causing different important diseases (Desvignes 1999; Hadidi et al. 2003).

PLMVd, an *Avsunviroidae* member of the *Pelamoviroid* genus, is frequently reported from peach and its hybrids. It is widely distributed in peach germplasm from Europe, Asia and America (Flores et al. 2003). The most characteristic symptoms caused by PLMVd in peach are deformation of fruits that usually are discoloured with cracked sutures and flattened stones. Furthermore, it has been occasionally detected in naturally infected plum, apricot, sweet cherry, cultivated and wild pear (Hadidi et al. 2003). Sequences of 335–342 nt and the presence of hammerhead structures in both polarity strands have been reported (Flores et al. 2003). PLMVd isolates (348–351 nt) known to cause peach calico (PC) were reported to have an extra sequence that contains the PC pathogenicity determinant (Malfitano et al. 2003). PLMVd appears as a species in which each isolate is a complex mixture of RNAs (Ambrós et al. 1998; Pelchat et al. 2000). Phylogenetic studies on PLMVd have indicated the presence of three main groups (Ambrós et al. 1998).

HSVd is a typical *Pospiviroidae* with a central conserved region (CCR) and without the hammerhead self-cleavage that belongs to the genus *Hostuviroid*. HSVd, the only member of the *Hostuviroid* genus, has been found in a wide range of hosts including hop, cucumber, grapevine, citrus, plum, apricot, peach, almond and pear (Shikata 1990; Sano 2003; Pallás et al. 2003). Specific disorders such as hop stunt (Shikata 1990), dapple fruit disease of plum and peach (Ragozzino et al. 2002; Sano et al. 1989), and citrus cachexia (Reanwarakorn and Semancik 1998, 1999) have been associated with HSVd infections. In other cases, the infection seems to be latent in some hosts such as grapevine (Shikata 1990) and apricot (Astruc et al. 1996). However, recently the association of HSVd with an apricot fruit disorder known as 'fruit degeneration' has been reported, characterized by fruit rugosity and the loss of organoleptic properties (Amari et al. 2007).

HSVd isolates have been divided, based primarily on overall homology, into three groups: plum-type, hop-type, and citrus-type (Shikata 1990). Recently, two additional groups probably derived from recombination events have been proposed (Kofalvi et al. 1997; Amari et al. 2001). In addition, it has been suggested that the previous hop-type group itself is likely to be the result of recombination between members of the plum-type and citrus-type groups (Kofalvi et al. 1997).

PLMVd has been reported to occur in the former Yugoslavia in peach and plum (Shamloul et al. 1995; Hadidi et al. 1997). However, this survey was very limited. Here, we carried out a large-scale survey (871 stone fruit trees representing 602 different cultivars) for the incidence of PLMVd and HSVd in several stone fruit collections and commercial orchards in Serbia, and the determination of sequence variants of both viroids from native and imported cultivars.

Materials and methods

Validation of sampling method for large-scale survey

Before starting the large-scale survey for viroid detection, a validation test was carried out to verify the distribution of PLMVd and HSVd within the infected trees in the open field. One-year old shoots, from two PLMVd-infected peach and two HSVd-infected apricots, were selected for each scaffold (three or four) in three levels of the canopy (basal, medium and top); for each shoot, three leaves were collected from bottom, medium and top positions. For each tree 27 or 36 leaves were collected depending to the number of scaffolds. The collected leaves were then processed simultaneously for comparative testing between tissue-imprint (TIH) and dot-blot hybridization (DBH), as described by Más and Pallás (1995).

Tissue-imprint (TIH) and dot-blot hybridization (DBH)

Leaf petioles were pressed onto a nylon membrane in four imprints for TIH. Total nucleic acids for DBH were extracted, as described by Menzel et al. (2002), from 0.5 g of leaf tissue. Hybridization and chemiluminescent detection of HSVd and PLMVd were performed as previously described (Pallás et al. 1998).

Large-scale survey for viroids by TIH

Stone fruit collections of the University of Belgrade, University of Novi Sad, and Fruit Tree Institute of Cacak, and several commercial orchards were visited in autumn 2004. The 871 stone fruit samples, represented by three leaves per tree, were randomly sampled from each tree. Collected samples represented 602 different cultivars of stone fruits: 189 of European plum

Table 1 Results of molecular hybridization for viroids

Species	Samples		Cultivars		Viroid infection
	I/T (no.)	Infection rate (%)	I/T (no.)	Infection rate (%)	
Peach	185/372	50	95/177	54	PLMVd
Plum	0/207	0	0/189	0	–
Apricot	2/111	2	2/96	2	HSVd
Sweet cherry	0/109	0	0/88	0	–
Sour cherry	0/44	0	0/44	0	–
Wild *Prunus*	0/28	0	0/8	0	–
Total	187/871		97/602		

I: infected, *T*: tested

(*P. domestica*), 177 of peach (*P. persica*), 96 of apricot (*P. armeniaca*), 88 of sweet cherry (*P. avium*), 44 of sour cherry (*P. cerasus*), and 8 wild *Prunus* spp. (Table 1). Leaf petioles were pressed onto a nylon membrane in three replicates during the field survey in Serbia. The membranes were then stored at 4°C, and processed two weeks later at the Mediterranean Agronomic Institute of Bari (Italy).

Nucleic acid extraction and viroid detection

Total nucleic acids were extracted, as described by Menzel et al. (2002) for nine PLMVd-infected peach cultivars and two HSVd-infected apricots (Table 3). For HSVd, the reverse transcription polymerase chain reaction RT-PCR was carried out as described by Astruc et al. (1996). Primers used were the VP-19 5′-d(GCCC CGGGGCTCCTTTCTCAGGTAAG)-3′, complementary to HSVd residues 60–85, and the VP-20 5′-d(CGCC CGGGGCAACTCTTCTCAGAATCC)-3′, complementary to HSVd residues 78–102. The PLMVd isolates were RT-PCR amplified with primer RF-43 5′-d(CTG GATCACACCCCCCTCGGAACCAACCGCT)-3′ and RF-44, 5′-d(TGTGATCCAGGTACCGCCGTAGAAA CT)-3′ (Ambrós et al. 1998). Primers RF-43 and RF-44 are complementary and identical to positions 208 to 178 and 199 to 225, respectively, of the PLMVd. Amplified products were analysed by electrophoresis in a 5% polyacrylamide vertical slab gel. Banding patterns were visualized by silver staining.

Cloning and sequence analysis

Amplified products were purified with QIAquick PCR purification Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and cloned in pGEMT Easy vector (Promega, Madison, WI, USA). Sequences of the recombinant plasmid were obtained by automatic sequencing (MWG Biotech, Ebersberg, Germany). Multiple alignments of nucleotide were obtained using the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment programme. Phylogenetic analysis was done using the minimum evolution method of phylogenetic inference (Rzhetsky and Nei 1993) with 10,000 bootstrap replicates. Version 2.1 of the Molecular Evolutionary Genetics Analysis (MEGA) software was utilized (Kumar et al. 2001). The nucleotide sequences were deposited in the GenBank database under the accession numbers indicated in Table 3.

Results

Validation of sampling procedure

The results of testing by TIH and DBH for PLMVd are shown in Fig. 1. Regardless of the tree, geographic orientation of the scaffold, level of the canopy, and position of the leaf in the shoot, 72 out of 72 leaf positions (100%) were positive by TIH and DBH. Similar results were obtained for HSVd (data not shown). This is an indication that PLMVd and HSVd are systemic and regularly distributed within the tree, in peach and apricot, respectively. Therefore, a large-scale survey for viroids by TIH and the use of three

Table 2 PLMVd infection distributed for peach cultivar origin

Cultivar origin	No. of cultivars		Infection rate (%)
	Tested	Infected	
Native Yugoslavia	4	4	100
Western Europe	57	33	58
Asia	12	7	58
North America	97	48	50
Unknown origin	7	3	43
Total	177	95	54

Table 3 List of sequenced viroid isolates

Viroid	Host	Cultivars	Origin	Variant	Length (nt)	Accession no.
PLMVd	Peach	Vineyard peach	Serbia	PL1	337	EF151292
PLMVd	Peach	Vineyard peach	Serbia	PL21	337	EF151293
PLMVd	Peach	Vineyard peach	Serbia	PL101	335	EF151296
PLMVd	Peach	Vineyard peach	Serbia	PL151	338	EF151300
PLMVd	Peach	Vineyard peach	Serbia	133A	337	EF151298
PLMVd	Peach	Maja	Serbia	PL112	337	EF151297
PLMVd	Peach	Tagiao	Japan	PL94	338	EF151295
PLMVd	Peach	Sinokuba	Japan	142A	337	EF151299
PLMVd	Peach	Kineska Plosnata	China	PL31A	338	EF151294
HSVd	Apricot	Saturn	Romania	427	294	EF151291
HSVd	Apricot	Karola	Czech Republic	56	297	EF151290

leaves collected from different parts of the canopy were considered to be reliable.

Large-scale survey for viroids

Surveys and sampling were made in 3 varietal collections and 33 commercial orchards. Fruits with suture cracking were observed in several PLMVd-infected peach cultivars, whereas only one peach tree showed calico symptoms. No particular symptoms were recorded in apricot trees tested positive for HSVd.

From 871 tested samples (372 peach, 207 plum, 111 apricot, 109 sweet cherry, 44 sour cherry and 28 wild *Prunus* spp.), 185 peaches were positive for PLMVd and two apricots for HSVd (Table 1). Among PLMVd-infected peach trees were also vineyard peaches originating from seeds. No cherries, plum and wild *Prunus* spp. were infected by the viroids. Ninety-five cultivars of peach (54% of the total) were infected by PLMVd and two of apricot by HSVd (2%) (Table 1). PLMVd-infected peach cultivars originated from the world's main breeding centre for this crop. Western European and Asian cultivars were the most infected (58%) followed by those in North America (50%) (Table 2).

Characterization of new PLMVd sequence variants

PLMVd was isolated from nine different peach sources, six local Serbian rootstocks or cultivars and three imported cultivars (Table 3). The length of the sequenced PLMVd isolates ranged between 335 and 338 nucleotides (Fig. 2), which was in agreement with previous reports on this viroid (Ambrós et al. 1998; Pelchat et al. 2000).

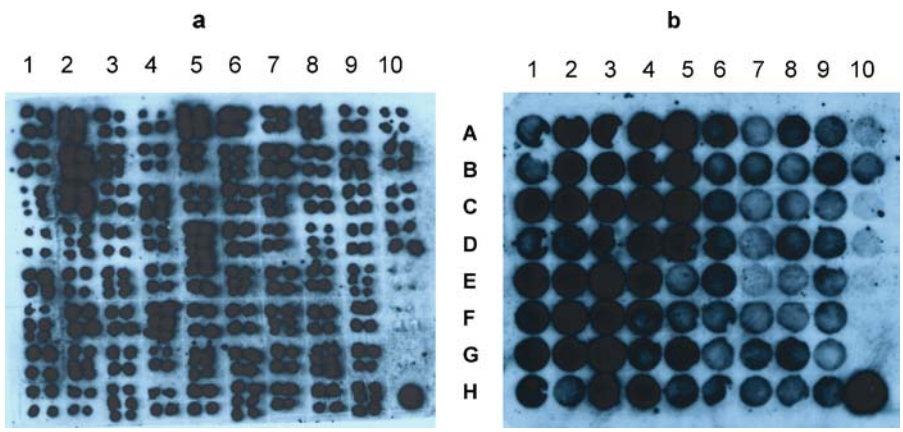

Fig. 1 Comparison of tissue-imprint (**a**) and dot-blot (**b**) hybridization for the detection of PLMVd. *Columns 1*, *2*, *3*, *4*, *5*, *6*, *7*, *8* and *9* are tested samples. *A*, *B*, *C* and *D* in column 10 are positive controls. *E*, *F* and *G* in column 10 are negative controls. In **a**, petioles were printed four times per square and in **b**, 3 μl of the total nucleic acids extracts from the corresponding leaf were deposited in the membrane. DNA plasmid control is spotted in column 10-H on both membranes

Fig. 2 Sequence alignment of nine molecular variants of PLMVd (in *bold letter*) analyzed in this work with 11 previously described variants from groups I (*blue*), II (*red*) and III (*black*). The consensus sequence of PLMVd in this alignment is shown at the *top*. The conserved nucleotides present in hammerhead structures are indicated on coloured background, *dark blue* and *red* colours refer to plus and minus polarities, respectively, and the self-cleavage sites are shown by *arrows*. The new base-pairing (G–U) between loops A and B detected in the PL1 variant is on *green* background. The pseudoknot between positions178–181 and 211–214 is indicated as *P8*

Molecular variability of PLMVd populations has been previously described (Ambrós et al. 1998; Pelchat et al. 2000). Ambrós et al. (1998) classified PLMVd isolates into three main phylogenetic groups (I, II and III). After the comparison between the sequences obtained from the isolates described in this work and the previously characterized ones, nine new PLMVd variants were identified (Fig. 2). All the obtained sequences clustered together in group III (Fig. 3); some of the new variants (PL1, PL84 and PL112, all from

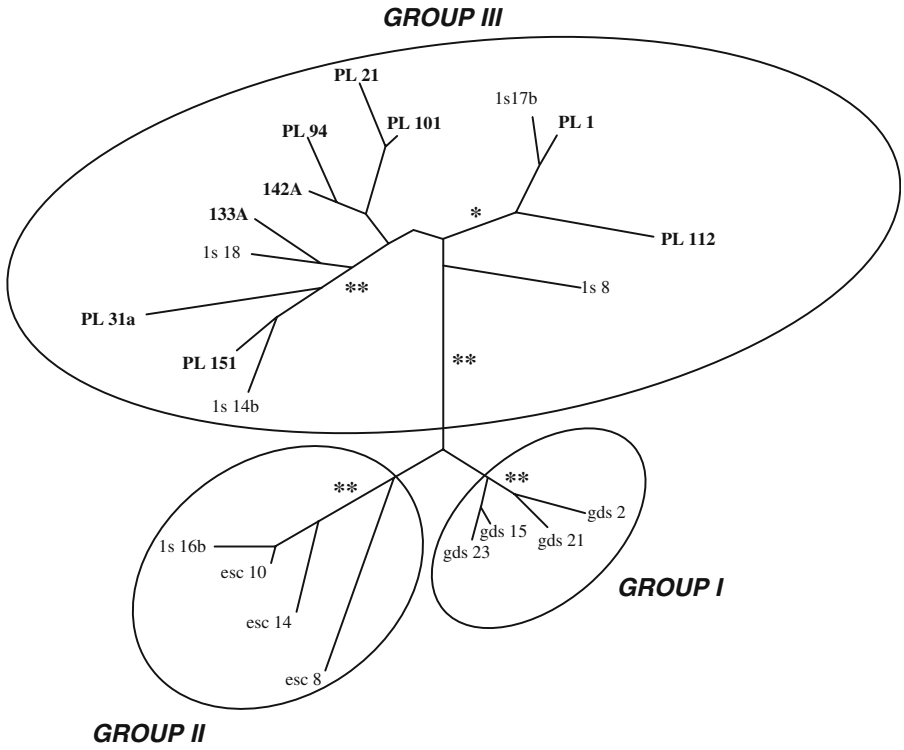

Fig. 3 Phylogenetic tree of the PLMVd variants. The analysis was based on the distance calculated between the nine sequence variants described in this work and 12 PLMVd sequence previously described. Three phylogenetic groups described by Ambrós et al. (1998) are shown. *Asterisks* indicate the statistical value of the node as determined by bootstrap analysis (10,000 replicates). **Node detected in >75% of replicates; *node detected in >50% replicates

Serbia) were very similar to the previously described variant 1s17b, whereas the rest were more similar to the variant 1s18, both coming from a latent isolate (Ambrós et al. 1998), close to the variants from isolate LS35 (Fig. 3). However, the informative changes, previously described to be characteristic of this group, were not strictly maintained in the PLMVd isolates characterized in this work. The U at position 5 as opposed to an A, present in members of the other two groups, was observed in only seven (PL1, PL151, PL31A, PL133A, PL142A and PL94A) of nine analyzed variant sequences. The presence of a C or A at position 2 was detected in these seven isolates as well as PL112. Variability was also observed in the U residue at position 339 and was present in only five isolates (PL151, PL31A, PL133A, PL142A and PL94) (Fig. 2). Interestingly the two variants of Japanese origin (PL94 and 142 A) were almost identical. It is also worthy to note that an important number of covariations were detected between positions 100 and 124 corresponding to the Pst arm of the molecule (see Fig. 3 in Ambrós et al. 1998), a highly structured region, reinforcing the secondary structure proposed for this region. Pseudoknot between positions 178–181 and 211–214 (P8 in Fig. 2) proposed by in vitro assays with nucleases and binding with oligonucleotides (Bussiere et al. 2000) was strictly conserved in all the nine new sequences described here. Regarding the pseudoknot-like element proposed by Ambrós et al. (1998) between loops A and B, we have found a new base-pairing (G_{341}–U_{65} in isolate PL1) not described previously that reinforce the structural relevance of this element.

Characterization of new HSVd sequence variants

HSVd was isolated from the two apricot cvs: Karola and Saturn, both of imported origin. The length of our sequenced HSVd was 297 and 294 nucleotides, respectively, for Karola and Saturn (Table 3), which was in agreement with previous reports on this viroid (Kofalvi et al. 1997; Amari et al. 2001).

Alignment (Fig. 4) and phylogenetic analysis (Fig. 5) of the two HSVd sequence variants obtained in this study showed that the HSVd variants isolated from apricot cv. Karola could be included in the previously recombinant group P-H/cit 3 (Kofalvi et al. 1997; Amari et al. 2001).

The four variants obtained from apricot cv. Saturn, showed three specifics changes in relation to the HSVd variants now characterized. These changes are

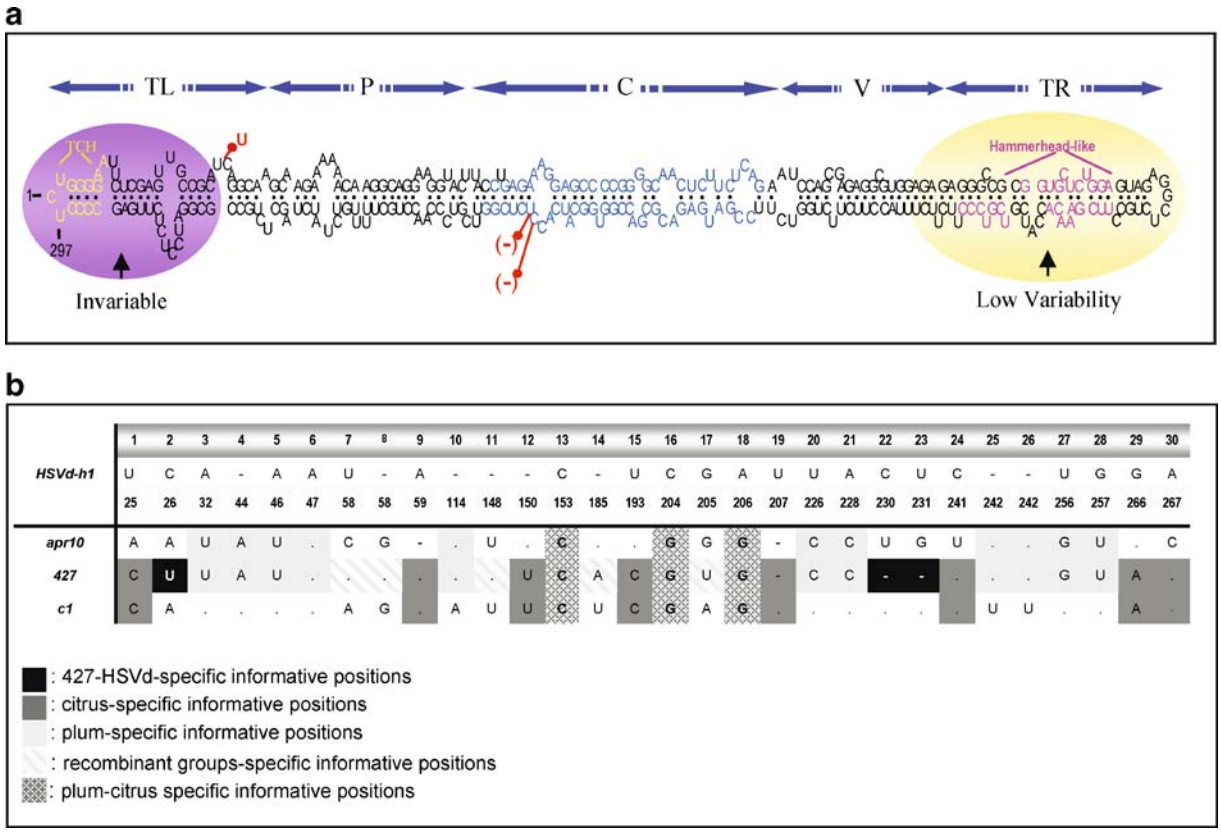

Fig. 4 **a** Predicted secondary folding of HSVd showing the phylogenetically informative changes. In *red* are marked the differences between Saturn-427 isolate versus reference isolate HSVd h1. **b** Condensed alignment of the 30 phylogenetically informative changes (Kofalvi et al. 1997) for the HSVd isolate analyzed in this work, the HSVd isolates *apr10* and *c1* were selected as reference sequences

a substitution (U/C) in position 26 and two deletions in positions 230 (C) and 231 (U). The modifications described in this sequence variant maintained the rod-like structure. The changes in positions 26 and 230 corresponded to loops and the unpaired residue resulting from deletion (U) 231 did not interfere with the proposed HSVd structure (Fig. 4). The three changes detected in the variant sequence consensus (designed as 427) were located in two different regions of the HSVd molecule, where a high degree of variability has been previously described. Only 30 of the so-called informative changes are required to discriminate among phylogenetic clusters of HSVd variants (Kofalvi et al. 1997). The analysis of the condensed alignment of these 30 informative changes versus those of a representative variant of the recombinant plum–citrus group (*apr* 10) and others from citrus group (c1) revealed that this new variant is composed of a mosaic of informative changes from both representative groups (Fig. 4) and therefore HSVd 427 can be considered the result of a new recombination event. In fact, phylogenetic analysis differentiated this new variant from within previously described groups (Fig. 5).

Discussion

The validation results indicated a similar detection sensitivity for TIH and DBH against PLMVd and HSVd, proving TIH was advantageous for the detection of stone fruit viroids. In addition, we proved that both PLMVd and HSVd are well distributed in the tree canopy of peach and apricot, respectively. As a consequence, even a single leaf collected randomly from an infected candidate tree will satisfy the needs to safely detect the viroids and avoid false negatives.

To our knowledge, this study for the presence of viroids is the largest survey ever done in the world for stone fruit viroids, testing 602 stone fruit cultivars. The

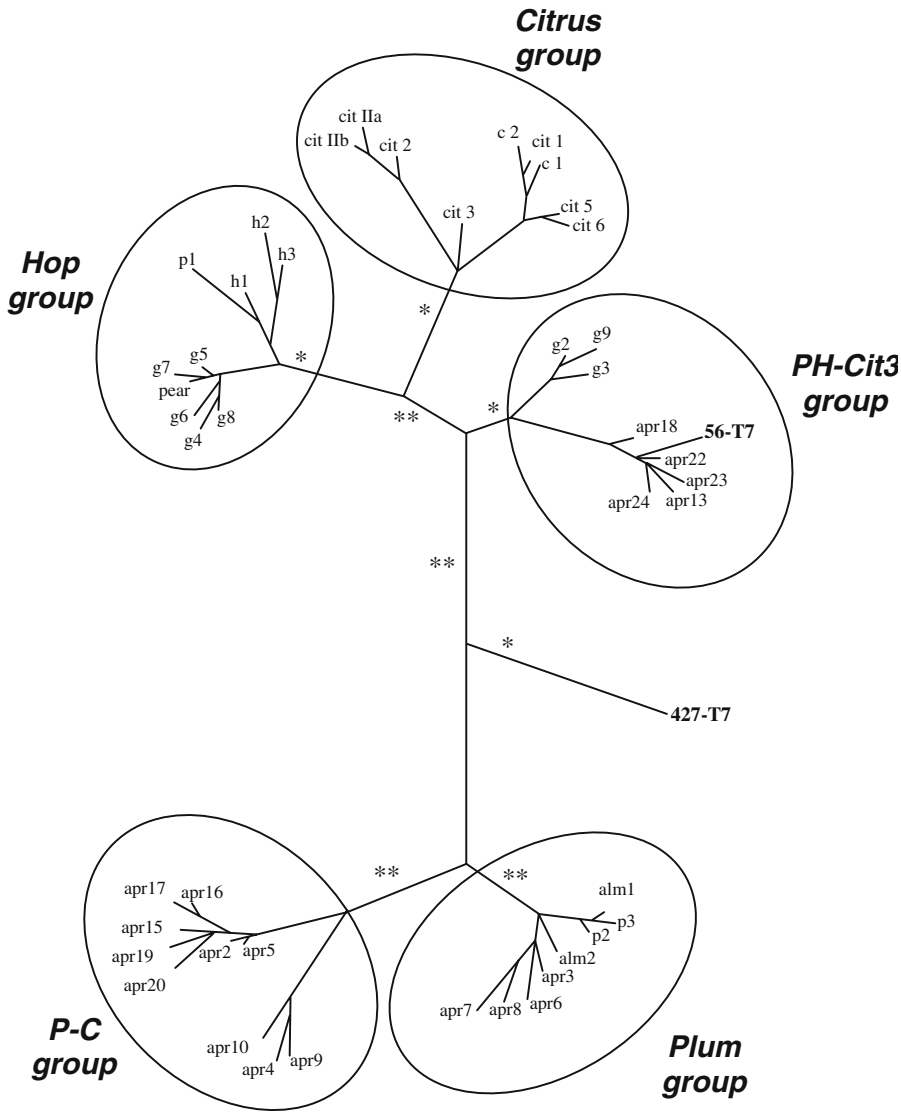

Fig. 5 Phylogenetic tree of HSVd sequence variants. Phylogenetic analysis was performed on genetic distance calculated between the HSVd variants described here (427and 56) and representatives HSVd sequences described previously. The five phylogenetic groups described by Amari et al. (2001) are delineated. *Asterisks* indicate the statistical value of the node as determined by bootstrap analysis (10,000 replicates). **Node detected in >75% of replicates; *node detected in >50% replicates

occurrence of HSVd is a new report for Serbia, whereas detection of PLMVd confirmed previous reports from former Yugoslavia in peach but not in plum (Shamloul et al. 1995; Hadidi et al. 1997). PLMVd was found in peach cultivars originating from the main world breeding centres for this crop. The high incidence of PLMVd in peach concurs with the data reported from other countries (Skrzeczkowski et al. 1996; Badenes and Llácer 1998; Hadidi et al. 1997; Michelutti et al. 2004).

The PLMVd-infected peach seedlings (vineyard peaches), reported by Matic et al. (2004) raise the problem of viroid epidemiology. However, there is no clear information on PLMVd epidemiology although the natural spread of the viroid in peach orchards has been reported (Desvignes 1986). Aphid- and cutting tool-transmission are possible infection routes (Desvignes 1986; Hadidi et al. 1997), but more studies are needed to clarify this aspect.

PLMVd shows a high level of molecular variability which appears, however, to be restricted with regard to the preservation of the branched conformation, the hammerhead structure (Ambrós et al. 1998; Pelchat et al. 2000; Flores et al. 2006) and the proposed kissing-loop interaction (Bussiere et al. 2000). All these constraints have been observed in the new variants from Serbia characterized in this study. In-

terestingly a new base pairing between loops A and B have been found in one of these variants.

All the obtained PLMVd sequences were clustered into one cluster (group III), independently of the peach cultivar analyzed and the geographical origin of the peach trees, suggesting a homogeneous phylogenetic origin of the Serbian isolates. However, the sequence of these Serbian isolates, in particular, and European ones, in general, cannot be differentiated from that of North American ones as previously indicated (Pelchat et al. 2000).

HSVd sequence variants from cv. Karola were clustered in the recombinant group P-H/cit 3 confirming that recombination events occur frequently between HSVd variants and that the intra-specific recombination could be a general mechanism in the evolution of viroids (Kofalvi et al. 1997; Amari et al. 2001). The three specific changes found in the variants of the isolate from cv. Saturn did not affect the rod-like structure. The three modifications detected in the same sequence variants, located in a high variable part of the molecule, reinforced the hypothesis that HSVd variability is restricted to certain polymorphic positions (Amari et al. 2001, 2007).

HSVd isolates from cv. Saturn were not clustered in one of the previously proposed groups, suggesting a novel putative group of HSVd isolates. The fact that all of the mutations observed in the sequence variant from the Saturn-427 isolate were coincident in the four clones analyzed allowed us to conclude that we obtained a high degree of fidelity in the characterization of the new sequence variants. The analysis of the so-called informative changes that discriminated among phylogenetic clusters of HSVd variants for the new Saturn-427, together with other representative sequence variants, suggest that it can be considered as a new recombinant cluster different from the previous ones (P-C and P-H/cit3 groups). However, an exhaustive analysis of more HSVd isolates from this region is necessary to support this hypothesis.

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