

SEQUENCE VARIABILITY OF *HOP STUNT VIROID* ISOLATES FROM STONE FRUITS IN TURKEY

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SUMMARY

Hop stunt viroid (HSVd), genus *Hostuviroid*, family *Pospiviroidae* is a circular single-stranded RNA 294-303 nt in size, infecting a large number of woody hosts, such as grapevine, *Citrus* spp. and *Prunus* spp. In this study, we report the identification and molecular characterization of HSVd variants isolated in Turkey from different naturally infected *Prunus* sources, including apricot, plum and peach. We determined the nucleotide sequences of eleven isolates and found five new sequence variants of 296 (3 variants) or 297 (2 variants) nt, comparable in length to previously known HSVd isolates. Multiple alignments and phylogenetic analyses showed that one apricot isolate (HSVd.AP1) clustered with the recombinant P-H/cit3 group, whereas all the others (one apricot; HSVd.AP23, two plum; HSVd.PL49 and HSVd.PL278 and one peach, HSVd.PE73) clustered with the Hop group, confirming the molecular variability of HSVd isolates. The sequence variability seems to be more related to the geographical origin of the isolates than to their hosts.

Key words: HSVd, detection, PCR, characterization, fruit trees, sequencing, viroid.

INTRODUCTION

Viroids are subviral pathogens whose genome consists of a small, circular RNA with a high degree of self-complementarity in its sequence (Diener, 1991). In some well characterized examples, viroid infection has been shown to cause plant diseases that adversely affect agronomic quality. *Hop stunt viroid* (HSVd) belongs to genus *Hostuviroid*, family *Pospiviroidae* and consists of 294-303 nucleotides. HSVd was first described as the causal agent of hop stunt disease in Japan and has since been found in several plant species, particularly in fruit

trees such as citrus, pear, peach, apricot, plum and almond (Shikata, 1990; Astruc *et al.*, 1996). In some hosts, such as grapevine (Shikata, 1990; Polivka *et al.*, 1996) and apricot (Astruc *et al.*, 1996), infection appears to be latent. Recently, HSVd was detected in degenerated apricot trees (Amari *et al.*, 2007). In other hosts, specific disorders such as hop stunt (Shikata, 1990), dapple fruit of peach and plum (Sano *et al.*, 1989; Ragozzino *et al.*, 2002) and citrus cachexia (Semancik *et al.*, 1988) have been associated with infection by HSVd. Overall sequence homologies (Shikata, 1990) and phylogenetic analyses (Hsu *et al.*, 1994) indicate that HSVd isolates can be separated into three main groups. Since isolates of each group had only a few isolation hosts, the groups were named plum-type (peach, plum and grapevine isolates), hop-type (hop, grapevine, peach and pear isolates) and citrus-type (citrus and cucumber isolates) (Sano *et al.*, 1989; Shikata, 1990). Recently, Kofalvi *et al.* (1997) characterized nine new HSVd variants from different *Prunus* sources, and the grouping of variants was redefined to include two new recombinant minor groups: plum-citrus type and plum-hop-citrus type (Pallás *et al.*, 2002).

Different studies were done to detect HSVd infection rates in different stone fruit species in Turkey. HSVd was detected with infection rates of 14.3%, 57.1%, and 66.6% in plum, peach and apricot, respectively (Torres *et al.*, 2004). However, HSVd was not detected in sweet or sour cherry samples from eastern Anatolia (Sipahioglu *et al.*, 1999). HSVd was also detected in an apricot sample from Turkey and its primary structure was determined (Amari *et al.*, 2000).

The studies conducted so far have been concerned with the western and eastern parts of Turkey and have not dealt with the molecular characterization of HSVd except for the apricot isolate (Amari *et al.*, 2001). In this study, a survey was made of the incidence of HSVd in several stone fruit orchards in the eastern Mediterranean region, and sequences of variant HSVd isolates were determined.

MATERIALS AND METHODS

Plant material. A total of 335 samples were collected

Table 1. HSVd distribution in orchards of the eastern Mediterranean region of Turkey.

Hosts	Samples	
	Infected/Tested	Infection rate (%)
Apricot	19/122	15.6
Plum	17/77	22.1
Peach	14/64	21.9
Sweet cherry	3/53	5.7
Almond	1/19	5.3
Total	54/335	16.1

from stone fruit orchards of the eastern Mediterranean region: 122 apricot, 77 plum, 64 peach, 53 sweet cherry and 19 almond (Table 1). Among the HSVd-infected trees, some HSVd isolates from different stone fruit sources were selected: six from apricot, four from plum and one from peach. Apricot isolates were designated as HSVd.AP1, HSVd.AP10, HSVd.AP23, HSVd.AP44, HSVd.AP67 and HSVd.AP68; plum isolates as HSVd.PL47, HSVd.PL48, HSVd.PL49 and HSVd.PL278; and the peach isolate as HSVd.PE73.

Detection of HSVd. Total nucleic acid (TNA) extraction was according to Astruc *et al.* (1996). Briefly, 0.5 g leaf tissue were triturated with a hand homogenizer inside sealed plastic bags in the presence of 5 ml extraction buffer (0.1 M Tris-HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl, 10 mM 2-mercaptoethanol). An aliquot (1 ml) of the homogenate was transferred to an Eppendorf tube, 50 µl of 20% SDS were added, and the sample was incubated at 65°C for 20 min, then mixed with 250 µl of 5 M potassium acetate and incubated on ice for 20 min. Samples were centrifuged at 12,000 rpm for 15 min, and the nucleic acids present in the supernatant fluid were recovered by ethanol precipitation and re-suspended in 40 µl sterile water.

Samples were analyzed for the presence of the viroid by RT-PCR, which was carried out using the primers designed by Astruc *et al.* (1996). For reverse transcription, 3 µl TNA, 1 µl complementary primer and 7 µl deionized

water were mixed. The mixture was heated for 5 min at 70°C and chilled on ice for 2 min. Nineteen µl of the reaction solution [4 µl of 5x first strand cDNA buffer (Fermentas GmbH, St. Leon-Rot, Germany), 2 µl 10 mM dNTPs (2.5 mM each), 0.5 µl RNase inhibitor (40 units/µl) (Fermentas), 12.5 µl de-ionized water] were mixed and held at 37°C for 5 min. One µl of cloned Moloney murine leukemia virus reverse transcriptase (200 units/µl) (Fermentas) was mixed with the annealing reaction mixture, which was then incubated at 42°C for 1 h, then at 72°C for 10 min. A 2 µl aliquot of the cDNA reaction served as template for PCR amplification. The PCR reaction consisted of 2.5 µl of 10 X PCR buffer (Fermentas), 2.5 µl 10 mM dNTPs (2.5 mM each), 1.5 µl MgCl₂ (25 mM), 0.3 µl Taq DNA polymerase (Fermentas) and 1 µl of each primer (20 pmol). Water was added to a volume of 23 µl. The reaction mix was denatured at 94°C for 1 min, followed by 30 cycles of denaturation at 95°C for 40 sec, annealing at 60°C for 40 sec and extension at 72°C for 1 min, with one final extension of 72°C for 5 min. PCR products (10 µl) were analyzed by electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized under an UV transilluminator.

Cloning, sequencing and molecular analyses. Amplified products were purified with Qiagen gel extraction kit and cloned in pGEM-Teasy plasmid vector (Promega, Madison, WI, USA). Sequences of the recombinant plasmid were obtained by automatic sequencing (ABI PRISM 310 Genetic Analyser, Perkin Elmer) by Iontek Sequencing Service (Turkey). Multiple alignment of nucleotide sequences of HSVd was obtained using the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment program. The alignment was used as input data to construct phylogenetic trees using the minimum evolution method of phylogenetic inference (Rzhetsky and Nei, 1993) with 10000 bootstrap replicates. Version 3.1 of the Molecular Evolutionary Genetics Analysis software MEGA version 3.1 was used (Kumar *et al.*, 2001). The nucleotide sequences were deposited in the GeneBank database under the accession numbers indicated in Table 2.

Table 2. HSVd sources used and representative HSVd sequence variants analyzed. New nucleotide changes are reported in bold.

Source	Isolate	Size (nt)	Closest HSVd sequence	Nucleotide differences with closest sequence	Accession Number
Apricot	AP1	296	apr.18	C105(-)	EF523826
Apricot	AP23	297	apr.21	A25G , T32A, (-) 44A, G54A, C260A	EF523827
Plum	PL49	296	apr.21	G54A, C106G, A266C	EF523828
Plum	PL278	296	apr.14	-106C	EF523829
Peach	PE73	297	apr.22	C25G , C26A, T32A, C105G , A266C	EF523825

RESULTS AND DISCUSSION

During the survey, different symptoms such as dwarfing and severe interveinal chlorosis were observed but samples were collected from both symptomatic and symptomless trees. Out of 335 samples tested, 19 apricot, 14 peach, 17 plum, three sweet cherry and one almond trees were found to be infected with HSVd. Table 1 shows the infection rates in stone fruit orchards of the eastern Mediterranean region.

The presence of HSVd in Turkey was first reported in the apricot cv. Septik by Amari *et al.* (2000). However, infection rates of HSVd in stone fruits were not studied in detail in the eastern Mediterranean region of Turkey. The infection rates detected in the current study ranged from 5.3% in almond to 22.1% in plum (Table 1). In eastern Anatolia, Sipahioglu *et al.* (2002) tested 148 stone fruit samples, mainly from native apricot, peach, sweet cherry and almond cultivars by dot-blot hybridization. None of the samples was found to be infected with HSVd and, more recently, only one apricot cv. Hacihaliloglu out of 491 stone fruit samples was positive to HSVd (Sipahioglu *et al.*, 2006). However, in western Anatolia the incidence of HSVd in apricot, peach and plum trees was 66.6%, 57.1% and 14.3%, respectively (Torres *et al.*, 2004). Our findings showed that viroidal infection rates detected in the eastern Mediterranean region were much higher than in eastern Anatolia for apricot, but lower than in western Anatolia for apricot and peach, though not for plum. The reason for differences in infection rates among the regions might be that different cultivars are grown in different regions. For instance, local cultivars largely prevail in eastern Anatolia where no new cultivars have been introduced, thereby preventing transmission from new inoculum sources.

HSVd was isolated from 11 different stone fruit sources: six apricots, four plums and one peach (Table 2). Genomic RNAs of the HSVd isolates sequenced were 296 or 297 nucleotide in size, in agreement with previous reports. Results of alignment and phylogenetic analyses of all HSVd sequences retrieved from the database showed that among the 11 Turkish isolates analyzed, there were five new HSVd variants. Isolates numbered 10, 44, 47, 67 and 73 had similar sequences and the same was found with isolates 48 and 49, while isolates 1, 23 and 278 had different sequences. Therefore, we chose five isolates designated as HSVd.PE73, HSVd.PL49, HSVd.AP1, HSVd.AP23 and HSVd.PL278 for further analyses. Table 2 shows the size, the closest HSVd variant, nucleotide differences from the closest published sequence, and GeneBank accession number of each of the newly sequenced Turkish HSVd isolates.

Figure 1 shows the sequence alignment of the five molecular variants of HSVd with the reference isolate HSVd.h1 (Ohno *et al.*, 1983). Isolates of the HSVd.PE73 group, all with 297 nt, were identified in apricot (isolate

number 10, 44, 67, 68), plum (isolate number 47) and peach (isolate number 73), thus showing that the sequence variability of HSVd isolates does not seem to be related to the host but, rather, to the region of origin, as previously reported (Ragozzino *et al.*, 2004). All isolates of HSVd.PE73 group were 98% identical to isolate apr.22 and show 10 phylogenetically informative changes compared with the reference isolate (HSVd.h1). Among the changes, those in position 25 (T-G) and 105 (C-G) were reported previously (grey box, Fig. 1). In particular, position 105 is a new phylogenetically informative change (Kofalvi *et al.*, 1997; Amari *et al.*, 2001).

Variant HSVd.AP23 was 297 nt in size and 98% identical to the closest isolate (apr.21) and showed seven changes compared to isolate h1, two of which (position 25 T-G and position 259 T-A, Fig. 1) had not been reported previously. In particular, the new informative change at position 25 is common to all sequences of the HSVd.PE73 group.

Both HSVd.PL49 (and consequently HSVd.PL48) and HSVd.PL278 variants had sequences of 296 nt that are 98% and 99% identical to the respective closest isolates (apr.21 and apr.14). HSVd.PE278 and HSVd.PL49 did not have new informative changes.

It should be emphasized that the only Turkish isolate (apr.14) already characterized from apricot is very similar (99% identity) to isolate HSVd.PL278 from plum, thus confirming the geographical origin of the HSVd molecular variants (Fig. 1). Isolate HSVd.AP1 is 296 nt in length, shares 99% identity to isolate apr.18, and has a deletion at position 105 (C) that has not been reported before (Fig. 1). All changes found in the five new HSVd variants were located in a segment with a high degree of variability and did not interfere with the proposed HSVd secondary structure. Furthermore, the changes do not interfere with the hypothesized hammerhead-like structure of HSVd, thus supporting its putative key role in viroid replication (Amari *et al.*, 2001). In conclusion, most of the variability for Turkish HSVd isolates was observed in the V and P domains. No variability was observed for the central region of the viroid molecule since the primers used were designed on this region.

Analysis of the informative changes required to discriminate among phylogenetic clusters of HSVd variants (Hsu *et al.*, 1994; Kofalvi *et al.*, 1997; Amari *et al.*, 2001), together with the phylogenetic tree, revealed that HSVd.AP1 variant clustered in the recombinant P-H/cit3 group, whereas all the other new HSVd variants found in this study clustered in the Hop group (Fig. 2).

In conclusion, this study has detected a 16.1% mean infection rate of HSVd in stone fruit trees in the eastern Mediterranean region. The clustering pattern of Turkish stone fruit HSVd isolates showed that sequence variability seems to be more related to the geographical origin of the isolates than to their hosts. Molecular characterization of the Turkish HSVd isolates confirmed the

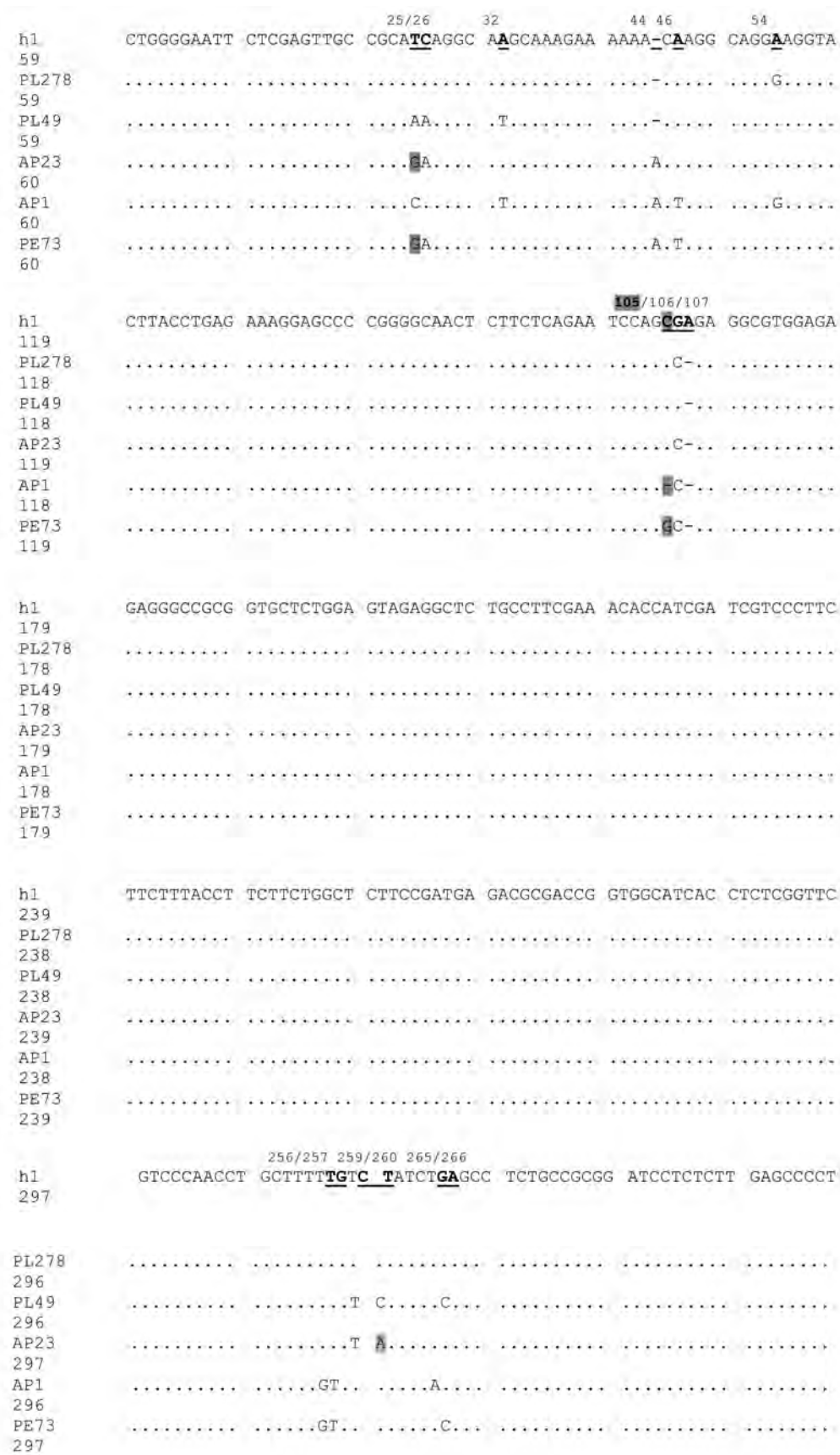


Fig. 1. Sequence alignment of five representative variants of HSVd analyzed in this work, compared with the reference isolate HSVd.h1 (Ohno *et al.*, 1983). Numbering of the positions of the phylogenetic informative changes is shown on the top, and relative original residues are underlined. The grey background indicates new specific changes not reported previously. Position 105 (in bold and grey box) represents a new polymorphic position.

existence of variability in the HSVd population and provided more information supporting the importance of infected plant materials in viroid distribution. This

calls for the enforcement of certification procedures for the production of viroid-free plants to be used in the establishment of new orchards.

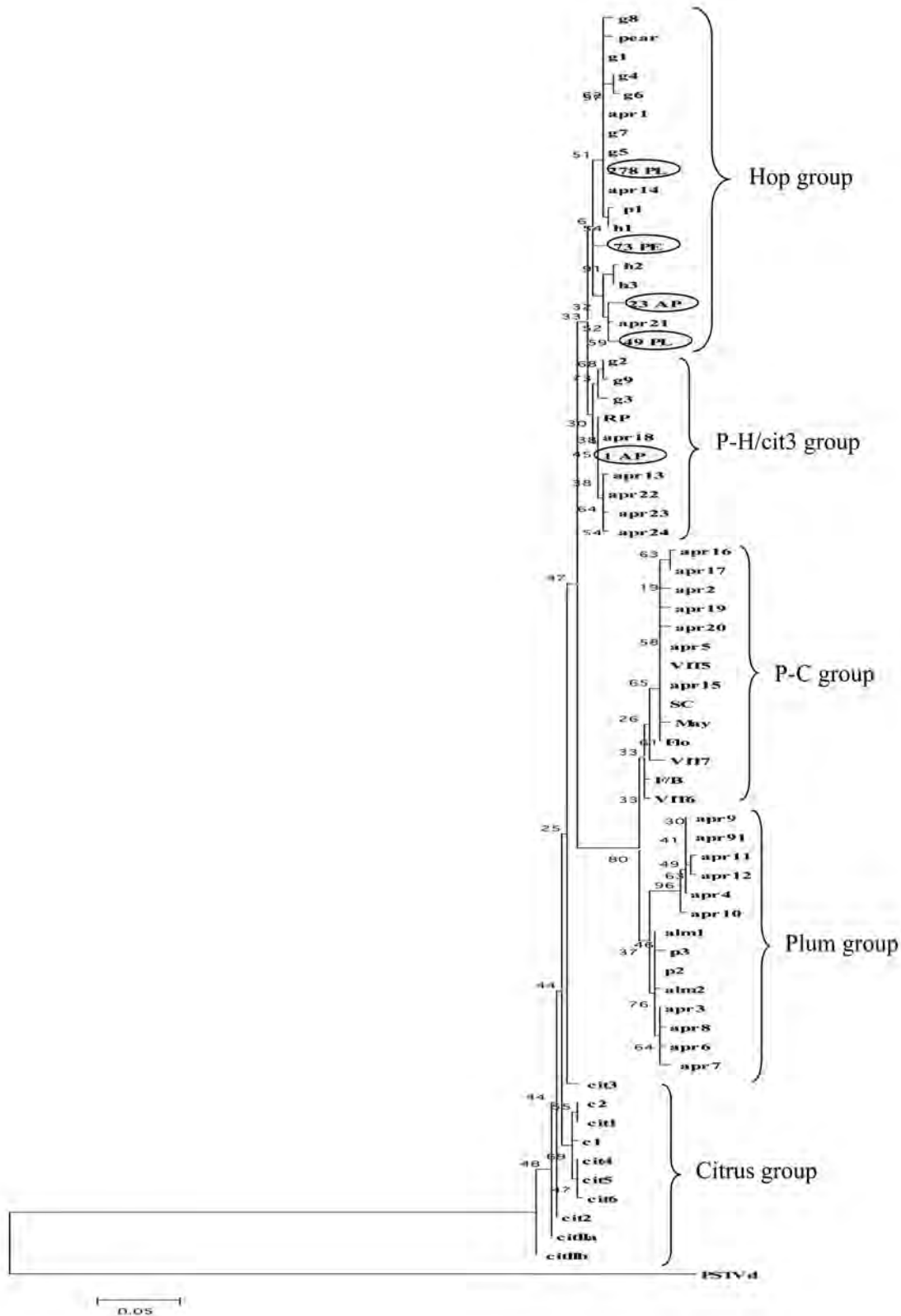


Fig. 2. Phylogenetic tree of HSVd sequence variants constructed using the minimum evolution method of phylogenetic interference with 10000 bootstrap replicates. Variants sequenced in this work are encircled. *Potato spindle tuber viroid* (PSTVd) was included as an outgroup. The five phylogenetic groups are shown, with bootstrap values shown on the branches.

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