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Molecular characterization and phylogenetic analysis of hop stunt viroid isolates from sweet cherry in China

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Abstract Hop stunt viroid (HSVd), a member of the family *Pospiviroidae*, infects a large number of plants and causes substantial economic losses. In this study, HSVd was detected in dappled fruits of sweet cherry and then molecularly characterized. We sequenced 164 cDNA clones from six sweet cherry cultivars in the Shandong Province of China and identified 23 HSVd sequence variants, which ranged in size between 293 and 303 nucleotides (nt) and shared 97~99% sequence identity with each other. Characterization of HSVd variants revealed that three were predominant in sweet cherry trees. Phylogenetic analysis showed that most of the HSVd variants isolated from sweet cherry were clustered within the plum-type group and that two variants were clustered with the recombinant P-C group, regardless of geographic origin. This study identified 23 HSVd variants and provided the molecular characterization of viroid infection (HSVd) in sweet cherry in China.

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Introduction

Viroids are the smallest pathogens, with genomes that consist of naked, single-stranded, covalently closed circular RNA molecules, ranging from 246 to 401 nucleotides (nt) in length, that do not code for proteins (Di Serio et al. 2014). Viroids can infect and systemically invade herbaceous and woody plants (Ding 2009; Kovalskaya and Hammond 2014). Based on their secondary structures and several biological features, they have been further classified into two families: *Pospiviroidae* and *Avsunviroidae* (Di Serio et al. 2014). Members of the *Pospiviroidae* family have the following five structural domains: terminal left (T_L), pathogenicity (P), variable (V), terminal right (T_R), and central (C) with a central conserved region (CCR) (Flores et al. 2005).

Hop stunt viroid (HSVd) is the type member of genus *Hostuviroid* of the family *Pospiviroidae*. HSVd was first described as the causative agent of stunt disease of hops in Japan, but since then, it has been found in various plant species, such as grapevine, citrus, cucumber, plum, peach, apricot, almond and jujube (Hataya et al. 2017). These plants showed specific disorders or symptoms, such as hop stunt (Shikata 1990), dappled fruits of plum and peach (Sano et al. 1989), cachexia of citrus (Semancik et al. 1988; Diener et al. 1988), pale fruits of cucumber (Lemmetty et al. 2011), and fruit degeneration of apricot (Amari et al. 2007). In other cases, such as for grapevine (Sano et al. 1985), almond (Canizares et al. 1999) and jujube (Zhang et al. 2009), HSVd infection appeared to be latent. In recent years, many

HSVd molecular variants have been reported and identified. For example, 10 molecular variants of HSVd from apricot, peach, and Japanese plum (Kofalvi et al. 1997), 16 sequence variants of HSVd from apricot (Amari et al. 2001) and 13 HSVd variants in pistachio (Elleuch et al. 2013) have been identified. In another study, 70 HSVd genomes from unique apricot and plum trees were sequenced, and 11 variants were identified (Jo et al. 2017). Sequence variants of HSVd have been divided into three major groups (plum-type, hop-type and citrus-type) and two minor groups (P-C-type and P-H/Cit-type) based on phylogenetic analyses (Shikata 1990). The two minor groups are probably derived from recombination events that occurred between members of the three main groups (Kofalvi et al. 1997).

Sweet cherry (*Prunus avium* L.) is an important economic fruit crop. Due to high returns, sweet cherry is gaining popularity in commercial orchards. As of 2016, the total planted area of sweet cherry was approximately 180,000 ha in China. Numerous sweet cherry cultivars have been developed and cultivated. Due to the clonal propagation of sweet cherry, the rates of virus and viroid proliferation in cultivated sweet cherry are very high. The viroid that infects sweet cherry trees has been reported to be associated with HSVd (Gazel et al. 2008; Xu et al. 2017), which has resulted in serious economic losses because it produces severe symptoms in sweet cherry fruits that render the fruits unmarketable. Although HSVd was identified in sweet cherry in China (Xu et al. 2017), there is limited information about the sequence variability and the phylogenetic relationships among HSVd variants. In the present study, we have identified and molecularly characterized 23 HSVd sequence variants from six sweet cherry cultivars collected from orchards in the Shandong Province to examine sequence diversity.

Materials and methods

Plant material

Fourteen sweet cherry samples with dappled fruit from six different cultivar sources ('Hongdeng', 'Tieton', 'Summit', 'Lapins', 'Zaoganyang', and 'Zhifuhong') were collected from orchards in suburban Taian, Shandong Province, China. Four of these samples were collected from 'Hongdeng', three from 'Tieton', three from 'Summit', two from 'Zhifuhong', and one each from

'Lapins' and 'Zaoganyang'. Fruit pericarp was harvested and frozen immediately using liquid nitrogen. All frozen samples were kept at −80 °C for further experimentation. The indicator plant cucumber (*Cucumis sativus* L.cv. Suyo) (Yang et al. 2008) was maintained in a greenhouse.

RNA extraction and sequence cloning

Total RNA was extracted from fruit tissue per the manufacturer's instructions using the EASYspin Plus Plant RNA Kit (AidLab, China). Reverse transcription polymerase chain reaction (RT-PCR) was used for cDNA synthesis per instructions from the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific, Beijing, China). The VP-19 (5′-GCCC CGGGGCTCCTTTCTCAGGTAAG-3′) and VP-20 (5′-CGCCCGGGGCAACTCTTCTCAGAATCC-3′) primers were used for PCR (Astruc et al. 1996); both primers lie in the strictly conserved central region of HSVd. For PCR amplification, high-fidelity DNA polymerase was used. PCR was carried out using 1 μl of cDNA, 1 μl each of the primers (10 μM), 1 μl of TransStart Fast Pfu Fly DNA Polymerase, 4 μl of dNTPs (2.5 mM), and 10 μl of buffer (TransGen Biotech, Beijing, China). Samples were placed in an Eppendorf Mastercycler Gradient 5331 thermocycler (Hamburg, Germany), and after an initial denaturing step at 95 °C for 5 min, the amplification profile consisted of 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 10 min. The PCR products (10 μl) were analysed via electrophoresis on a 1.5% agarose gel. The amplified RT-PCR products were purified and ligated into the pEASY-Blunt Cloning Vector (TransGen Biotech, Beijing, China) and then used for the transformation of *Escherichia coli* strain DH5α. Recombinant DNA clones were subjected to PCR to verify the presence of an insert of the expected size. At least eight randomly selected clones from each of the recombinant plasmid constructs were subjected to Sanger sequencing.

Biological indexing

Cucumber (*C. sativus* L.cv. Suyo) was used as an indicator plant. Total RNA was extracted from infected sweet cherries cv. 'Hongdeng' from the field and used to inoculate experimental plants via a standard buffer

(100 mM Tris-HCl, 100 mM EDTA, pH 7.5) and a conventional carborundum method. Cucumber seedlings were mechanically inoculated at the cotyledon stage. The seedlings were mock-inoculated with buffer as the healthy controls. Inoculated and healthy plants were grown in a greenhouse at 28~30 °C with 16 h photoperiods. Six weeks' post-inoculation, total RNAs were extracted and assayed for the presence of HSVd by RT-PCR using the VP-19/VP-20 primer pair as described above.

Phylogenetic analysis and secondary structure of HSVd sequences

Multiple alignment of HSVd sequences was performed by ClustalW using MEGA6.0. A phylogenetic tree was constructed employing the neighbour-joining method and tested with 1000 bootstrap replicates using MEGA6.0. The secondary structures of the HSVd genomes were predicted with the Mfold program (Zuker 2003) and modified with the RnaViz program (De Rijk et al. 2003).

Results

Identification of HSVd from sweet cherry

Using HSVd-specific primers in conjunction with RT-PCR, we examined 14 sweet cherry samples that had dapple symptoms (Fig. 1a), and amplicons of the expected 300 nt size were obtained (Fig. 1d, e). From each sample, at least eight clones were sequenced, resulting in a total of 164 HSVd genome sequences. The length of all sequenced HSVd variants ranged between 293 and 303 nt. Typical symptoms of stunting and reduction in flower diameter were observed in cucumber infected with total RNAs extracted from cv. 'Hongdeng' (Fig. 1c, g); HSVd was detected by RT-PCR in the infected cucumber plants approximately six weeks post-inoculation (Fig. 1f).

Characterization of HSVd sequence variants

Twenty-three HSVd sequence variants were identified from 164 genome sequences that were obtained from these isolates. All the sequences had 97~99% similarity among them, indicating that HSVd variants isolated from sweet cherry constitute quasispecies (Flores et al. 2014).

We then compared these HSVd variants with previously submitted HSVd sequences in GenBank. The sequence comparisons revealed a high identity to HSVd variants deposited in GenBank. Four variants (SC-1, SC-2, SC-3, and SC-12) were found to be identical to previously reported variants, while 19 sequences were new variants from sweet cherry; 15 of the 19 variants were detected only once, and the other four variants appeared more than twice. Table 1 lists the cultivar of sweet cherry, number of isolates, size, the closest related HSVd variant, and nucleotide differences with the closest published sequence.

The incidence of each variant sequence was variable in each cultivar. For example, HSVd variant SC-1 was detected in all cultivars but was dominant in 'Tieton', 'Summit', 'Lapins', and 'Zhifuhong'; SC-2 was not detected in 'Tieton' and 'Lapins' but was detected in all the others and was dominant in 'Hongdeng' and 'Zaoganyang'; and SC-3 was not detected in 'Zhifuhong' and 'Lapins' but was detected in all the rest. We examined the number of sequenced HSVd clones for all samples and found that variant SC-1 was predominant (76 genomes), followed by SC-2 (32 genomes), and then SC-3 (23 genomes) (Table 1). Based on the number of each variant, SC-1 represented 46% of the genomes, followed by SC-2 (20%), and SC-3 (14%). The three dominant variants represented 80% of all HSVd variants isolated from sweet cherry.

Sequence analysis and secondary structure prediction

In comparison to the genome sequence of the first reported HSVd (GenBank: X00009) (Ohno et al. 1983), these 23 HSVd variants from sweet cherry (Table 1) shared 92~94% similarity, and a consensus sequence identical to variant SC-1 was obtained by aligning the 23 variant sequences. Using this consensus sequence, the secondary RNA structure was predicted using the Mfold program. The predicted structure with the lowest minimum free energy was a branched rod-like structure (Fig. 2). When compared to the secondary structure of the hop strain of HSVd (GenBank: X00009), the consensus sequence formed a more stable, rod-like secondary structure (dG = −110.00) (not shown). The nucleotide differences among the sweet cherry variants were further analysed. Twenty-eight variable sites of the 297 positions were identified, of which 11 were present more than four times, and mutation and
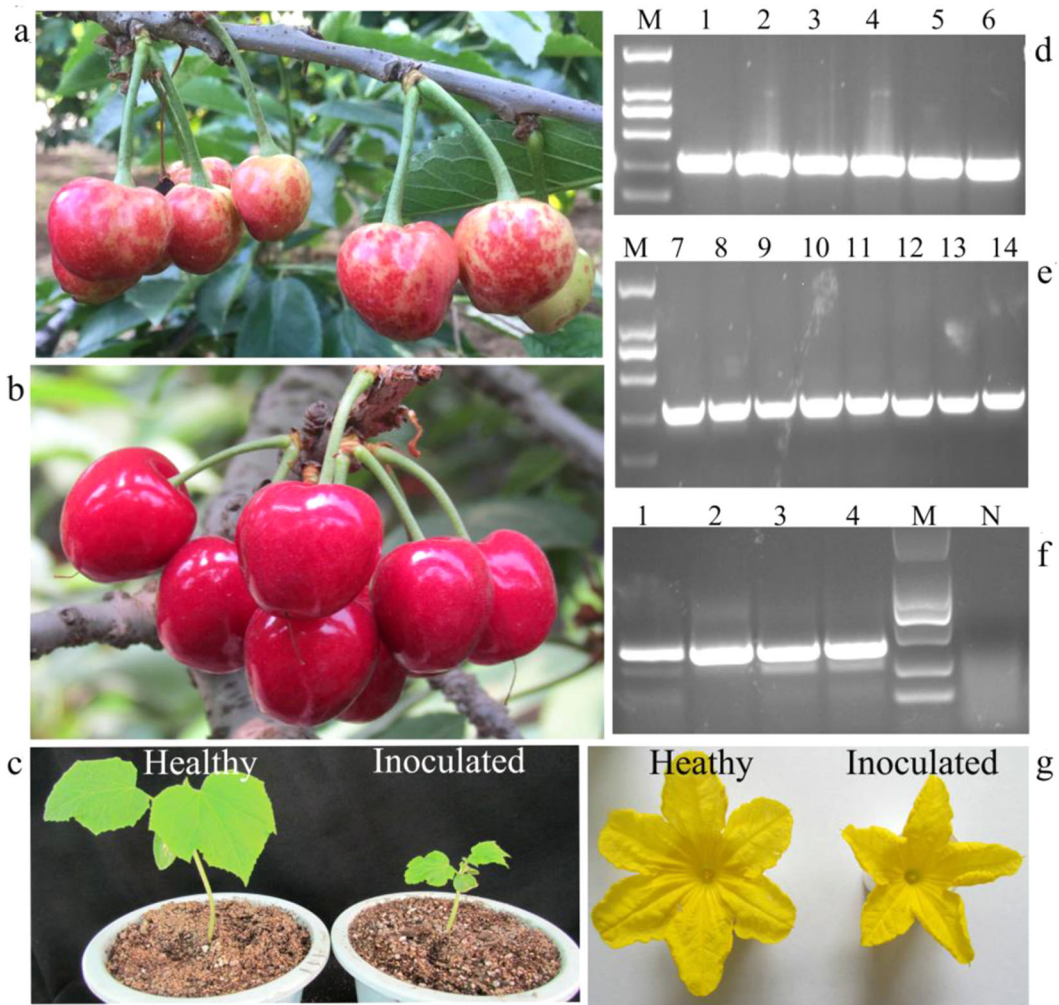
Fig. 1 Biological and RT-PCR detection of HSVd in infected plants. **a**: HSVd-infected sweet cherry tree cv. 'Zhifuhong' in the field showing dappled fruit. **b**: Healthy sweet cherry tree cv. 'Zhifuhong' in the field. **c**: Inoculated cucumber cv. 'Suyo' showing stunting after mechanical inoculation with HSVd-infected sap from sweet cherry cv. 'Hongdeng'. **d**–**e**: RT-PCR detection of HSVd in 14 samples of sweet cherry as described in Materials and methods; M, DL2000 marker; 1–14, sweet cherry samples. **f**: RT-PCR detection of HSVd in cucumber plants; M, DL2000 marker; N, Mock-inoculated cucumber; 1–4, inoculated cucumbers. **g**: The flower of an inoculated cucumber plant

indel sites were present in five domains of the HSVd secondary structure (Fig. 2). In contrast to some previous reports, there were changes in the CCR domain in some of the sweet cherry HSVd variants, in agreement with another study that also found that two nucleotides in CCR were variable among HSVd variants (Jo et al. 2017). However, the functional role of these domain variants remains unknown. We also found that the nucleotide changes affect neither the terminal right region nor the secondary structure, except in variants SC-3, SC-7, SC-8, and SC-10 (data not shown).

Phylogenetic analysis of HSVd variants

We collected all known HSVd sequences from the NCBI GenBank database using "Hop stunt viroid" as a query, and a total of 773 sequences were found. Duplicate and partial sequences were then removed, and we performed blast searches to identify sequences that had been incorrectly annotated. Of the 773 HSVd sequences, 458 variants were identified. We aligned the complete nucleotide sequences of the 458 variants using the ClustalW program. Then, the 23 HSVd variants characterized in this study and 49 representative HSVd

Table 1 The sources and sequence variants of HSVd from sweet cherry analyzed

Isolate (GenBank)	Cherry cultivars	No.of isolates	Size (nt)	Closest sequence (GenBank)	Plant species	Nucleotide differences with closest sequence
SC-1 (KY399984)	Hongdeng, Tieton, Summit, Lapins, Zaoganyang, Zhifuhong	76	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	Identical
SC-2 (KY399985)	Hongdeng,Zaoganyang, Summit, Zhifuhong	32	297	AF (D13764.1)	peach (Japan)	Identical
SC-3 (MH537073)	Hongdeng, Tieton, Summit, Zaoganyang	23	297	FP1 (AB098500.1)	plum (Japan)	Identical
SC-4 (MH537074)	Tieton	1	297	AF (D13764.1)	peach (Japan)	A36G
SC-5 (MH537075)	Zaoganyang	1	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	T18C
SC-6 (MH537076)	Summit, Zhifuhong	3	303	apr3 (Y09345.1)	Apricot Bulida (Spain)	(--)8AG;(--)145CG;(--)225CA
SC-7 (MH537077)	Zhifuhong	1	303	apr3 (Y09345.1)	Apricot Bulida (Spain)	(--)8GA;(--)145CC;(--)225GG
SC-8 (MH537078)	Summit	1	298	apr3 (Y09345.1)	Apricot Bulida (Spain)	(-)46A
SC-9 (MH537079)	Hongdeng	2	303	FP1 (AB098500.1)	plum (Japan)	(--)9GA;(--)146AA;(--)225CG
SC-10 (MH537080)	Hongdeng	1	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	G223A
SC-11 (MH537081)	Hongdeng	1	297	AF (D13764.1)	peach (Japan)	C164T
SC-12 (MH537082)	Lapins	1	297	FP2 (AB098502.1)	plum (Japan)	Identical
SC-13 (MH537083)	Summit	1	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	A104C
SC-14 (MH537084)	Tieton	1	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	A225G
SC-15 (MH537085)	Summit	1	293	apr3 (Y09345.1)	Apricot Bulida (Spain)	CTTC91(----)
SC-16 (MH537086)	Zhifuhong	1	297	AF (D13764.1)	peach (Japan)	C226T
SC-17 (MH537087)	Tieton	1	296	apr3 (Y09345.1)	Apricot Bulida (Spain)	A88(-);T205G
SC-18 (MH537088)	Hongdeng, Summit, Lapins	8	303	apr3 (Y09345.1)	Apricot Bulida (Spain)	(--)8AG;C81(-);(--)145AA;G198A;(-)225CG
SC-19 (MH537089)	Tieton	4	298	apr3 (Y09345.1)	Apricot Bulida (Spain)	(--)67C;T207G
SC-20 (MH537090)	Summit	1	303	apr3 (Y09345.1)	Apricot Bulida (Spain)	(--)8AG;(--)145AA;G-A;(--)225CG
SC-21 (MH537091)	Tieton	1	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	T10C;T179C
SC-22 (MH537092)	Tieton	1	297	AF (D13764.1)	peach (Japan)	A209G
SC-23 (MH537093)	Zaoganyang	1	297	apr3 (Y09345.1)	Apricot Bulida (Spain)	T220C

(-) and (- -) represents missing nucleotide

sequences selected based on the first phylogenetic analysis were subjected to a second phylogenetic analysis. PSTVd clone PSTVd_3 (KF418767.1) was used as the outgroup. Using the grouping classification of previous reports, HSVd variants were divided into five groups (Fig. 3). Twenty-one variants from this study belonged to the plum-type group, and two variants were included in the P-C recombinant group, which previously contained the variants HSVd.apr2 and HSVd.apr5 (Kofalvi et al. 1997; Amari et al. 2001).

Discussion

HSVd has a broad host range and has been detected and characterized in peach, apricot, almond, pear, plum, grapevine, hop, citrus, sweet cherry, and jujube in China (Li et al. 2006; Yang et al. 2006; Zhou et al. 2006; Rizza et al. 2007; Yang et al. 2007; Guo et al. 2008; Mu et al. 2009; Zhang et al. 2009; Wang et al. 2010; Xu et al. 2017). In this study, we focused our attention on sweet cherry trees. Fourteen samples with dappled fruit were
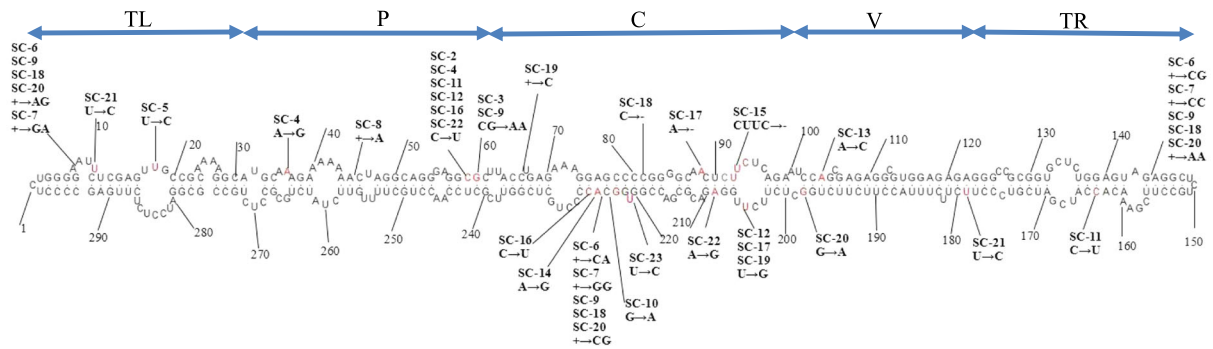
Fig. 2 Secondary structure predicted by the Mfold program using the HSVd consensus genome sequence from sweet cherry. The specific sequence variations of the other 22 HSVd variants were plotted on the predicted secondary structure. Number indicates the position of the nucleotide. The terminal left (TL), pathogenicity (P), central conserved (C), variable (V), and terminal right (TR) domains are designated based on a previous study (Amari et al. 2001)

collected from six sweet cherry cultivars. RT-PCR amplification products ranged between 293 and 303 nt, in agreement with previous reports on the size of this viroid (Zhou et al. 2006; Zhang et al. 2009). Twenty-three HSVd variants in sweet cherry were identified, of which four were identical to previously reported HSVd variants, while the remaining 19 variants had not been reported before. It has been reported that viroid variants with 2~5% divergence existed even within one host (Lin et al. 2015). A comparison with the closest HSVd sequences in GenBank revealed that 19 HSVd sequences had 1~5 nucleotide changes. Variant SC-1 was found to be identical to HSVd.apr3 isolated from apricot plants (cv. Bulida) in Spain (Kofalvi et al. 1997), and 14 variants were 99% homologous to HSVd.apr3. Variant SC-2 was identical to the HSVd variant AF from dapple fruit disease of plum and peach in Japan (Sano et al. 1989) and was identical to five variants isolated from *Prunus persica* and *Prunus mume* (Jo et al. 2017). Similarly, variants SC-3 and SC-12 were 100% identical to HSVd variants FP1 and FP2, respectively, from dapple fruit disease of plum in Japan. These combined results suggest that the same or similar HSVd variants could be isolated from different cultivars of the same host or even from different hosts from different regions.

Viroid-infected plants can be asymptomatic or develop symptoms ranging from mild to severe, depending on viroid strains and host species (Owens et al. 1995, 1996). Characterization of HSVd variants revealed that three predominant variants (SC-1, SC-2, and SC-3) were present in sweet cherry trees in China. Variant SC-1 was the most dominant, accounting for approximately 50% of the total number of all variants. Variant

SC-1 could be detected in all cultivars of sweet cherry, whereas variants SC-2 and SC-3 could not be detected in two cultivars. In addition, mutational analysis showed that variant SC-2 differed from variant SC-1 at position 59 (C to U), while variant SC-3 differed from variant SC-1 at positions 59 and 60 (CG to AA). These two nucleotide positions showed high mutation rates, suggesting that HSVd genomes in this study may be derived from variant SC-1. The nucleotide differences in the pathogenicity and variable regions of *Pospiviroidae* play important roles in modulating symptom expression (Sano et al. 1992; Sano and Ishiguro 1998). In this study, the nucleotide changes of the dominant variants were in the 5′ end of the pathogenicity domain, which might be responsible for the expression of symptoms. The results showed that the three variants were unequally distributed in all the samples analysed, indicating that the dappled fruit symptoms may be correlated with one or two HSVd variants. However, further experiments are required to confirm which mutated position can influence symptoms of HSVd infection in sweet cherry.

In the phylogenetic analysis, 23 HSVd variants were compared with selected worldwide representatives, and five groups of HSVd genomes were revealed. The results showed that 21 HSVd variants clustered with HSVd.apr3, HSVd.apr7, HSVd.p2, and HSVd.p3, which belong to the plum-type group. The other two variants, SC-3 and SC-9, clustered together with HSVd.apr2 and HSVd.apr5, which were in the group P-C, resulting from a recombination event between plum-type and citrus-type isolates (Kofalvi et al. 1997). There was no correlation among geographical region, host, and genetic variation. Further analysis

Fig. 3 Phylogenetic relationships of HSVd sequence variants. Variants sequenced in this work are indicated with a black triangle. The position and branching of PSTVd_3 is included in this analysis as an outgroup. The five phylogenetic groups identified are shown. Bootstrap (confidence values) are shown at the branch points (1000 replications) and branch lengths are proportional to the number of inferred character state transformations
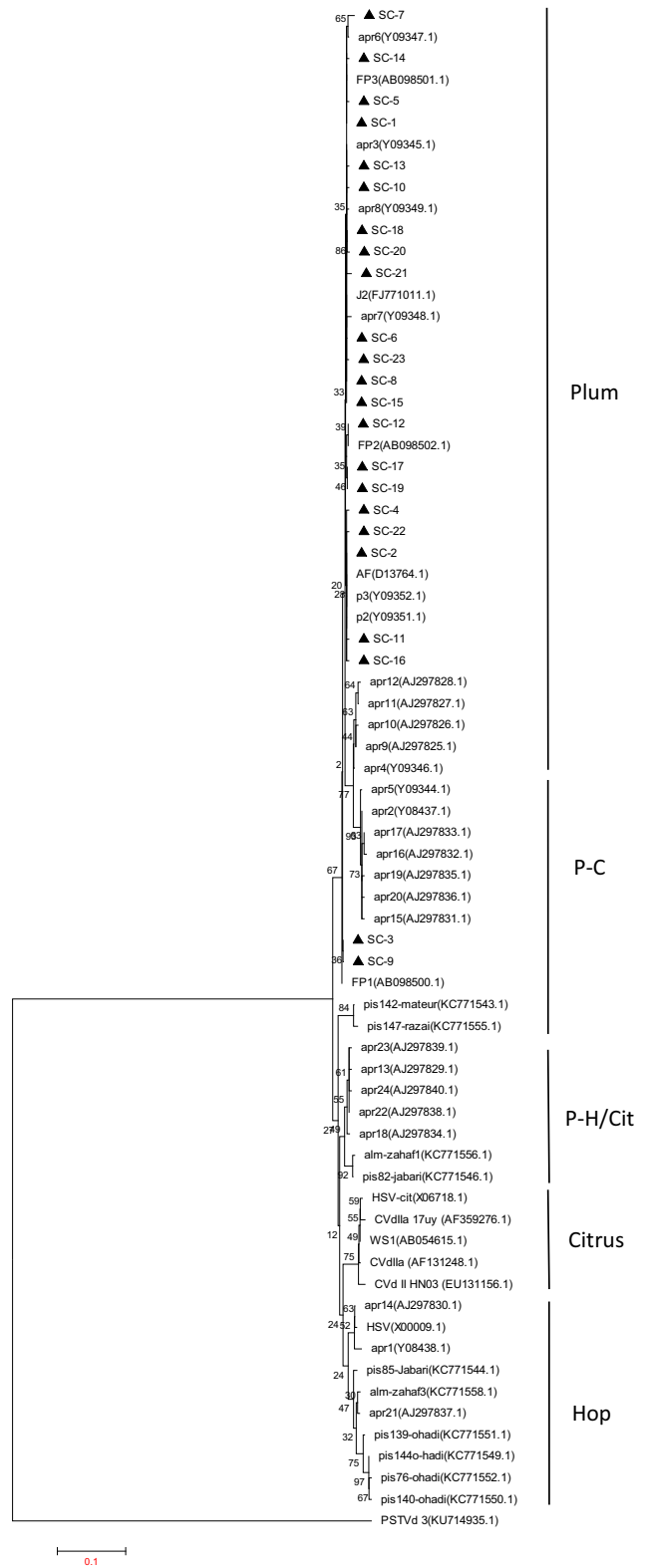
showed that a C base was located at informative position 7 (positions 58 of HSVdh1) in HSVd.apr3, HSVd.apr4, HSVd.apr6, HSVd.apr7 and HSVd.apr8, and a U in HSVd.p2 and HSVd.p3. In HSVd.apr2 and HSVd.apr5, the AA residues were at informative positions 7 and 9 (positions 58 and 59 in HSVdh1, respectively) (Kofalvi et al. 1997). In this study, two variants (SC-3 and SC-9) had AA bases at positions 59 and 60, while the other 21 variants had a C or a U base at position 59. These results were consistent with those of a previous study (Kofalvi et al. 1997), suggesting that the two informative sites could account for the clustering of HSVd variants.

The finding of multiple HSVd variants in all sampled sweet cherry trees whose fruits exhibited dappled fruit symptoms suggests that HSVd may be widespread in the Shandong province, the most important sweet cherry production area in China. To control the introduction and spread of the HSVd, certification of nursery stock using sensitive detection methods, decontamination of pruning tools, and selection of tolerant or resistant varieties (Rubio et al. 2016) are strategies that can reduce crop losses.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and/or animals rights We confirm that in this research any human and/or animals participant was not used and there is no any disagreement with informed consent.

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