

## First report and molecular analysis of *Apple scar skin viroid* in sweet cherry

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### Abstract

*Apple scar skin viroid* (ASSVd) is a serious pathogen of pome fruits. Recently, it has been reported in Chinese apricot and Chinese peach. In the context of our research on fruit tree viroids in Greece, ASSVd was initially detected in a sweet cherry tree cv 'Tragana Edessis' from Florina (Macedonia) by RT-PCR and this finding was confirmed by direct sequencing. This tree is located at the edge of a newly established apple orchard, along with other sweet cherry and wild cherry (*Prunus avium*) trees. In order to verify this interesting finding, we examined for ASSVd four sweet cherry trees, two wild cherry trees and their neighboring apple trees in the same orchard.

The examination was done by imprint hybridization using an ASSVd-specific DIG-labelled probe at stringent hybridization conditions and by RT-PCR using two different ASSVd specific primer pairs. We obtained ASSVd-positive results for all 6 cherry trees. No ASSVd was detected in the apple trees of the orchard. Purified ASSVd-positive RT-PCR products from the cherries were directly sequenced or cloned into the pGEM-T vector and then sequenced. ASSVd sequences were obtained from 5 trees. These sequences are 327-340 nucleotides long and share 96-99% identity with ASSVd isolates from Asian (Indian) apples. These results are similar to our data for other ASSVd variants from cultivated and wild pome fruit trees in Greece.

The cherry ASSVd variants differ from the ASSCS prototype isolate of ASSVd at 18-29 sites. There are 15 nucleotide changes (differences from ASSCS) common to all Hellenic ASSVd variants, including cherry and pome fruit tree variants. There are no cherry-specific nucleotide changes in the ASSVd sequences obtained. To our knowledge, this is the first published report of natural infection of cherry by ASSVd.

Keywords: ASSVd, cherry, molecular analysis, Hellenic sequences

### Introduction

*Apple scar skin viroid* (ASSVd) is the type species of the genus *Apscaviroid* (family *Pospiviroidae*). This 330 nt-long viroid induces serious diseases on pome fruit trees, such as apple scar skin, dapple apple, pear rusty skin and pear dimple fruit in Europe, Asia and North America (Hashimoto and Koganezawa, 1987; Hadidi et al., 1990; Zhu et al., 1995; Osaki et al., 1996; Koganezawa et al., 2003; Kyriakopoulou et al., 2003; Shamloul et al., 2004; Hadidi and Barba, 2010). It has been reported in apple (*Malus domestica*), pear (*Pyrus communis*, *P. pyrifolia*), wild apple (*M. sylvestris*) and wild pear (*P. amygdaliformis*) (Kyriakopoulou and Hadidi 1998; Kyriakopoulou et al. 2001, 2003; Koganezawa et al. 2003; Boubourakas et al. 2008). Recently, it was reported in Chinese peach and apricot from Sinkiang (Zhao and Niu 2006, 2008).

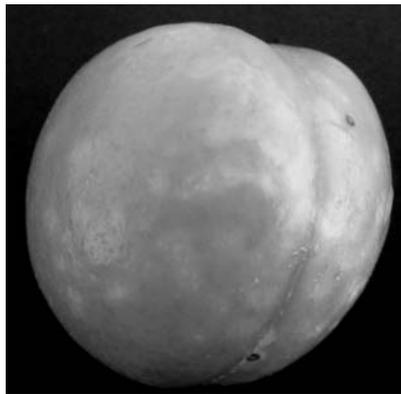
### Materials and methods

**Sampling, extraction, RT-PCR:** During a survey for viroids in Greece in 2008, 11 apple and 2 sweet cherry (*Prunus avium*) samples from a newly established apple orchard in Florina (Macedonia region) were tested by RT-PCR, using ASSVd and HSVd specific primers, respectively. The sweet cherry trees showed mosaic symptoms on leaves and white spots on fruit (Fig. 1-3). After ASSVd-positive results, a second sampling took place in the same orchard; 6 wild and cultivated (cv. 'Tragana Edessis') sweet cherry tree samples, all being at the edge of the orchard, and all their neighboring apple trees were tested for ASSVd, by imprint hybridization using an ASSVd-specific DIG-labelled probe at stringent hybridization conditions (50% formamide, T=60°C).

Five trees were tested by one tube, two step RT-PCR (Faggioli et al. 2001) using two different ASSVd-specific primer pairs (Hadidi and Yang 1990; Di Serio et al. 2002) and another one with one ASSVd-specific primer pair (Di Serio et al. 2002). In addition, 6 apple samples from the orchard were sent to the Phytopathology Laboratory, Hirosaki University (Japan) for further examinations by PAGE and Northern hybridization, using DIG-labelled ASSVd, ADFVd and AFCVd- specific riboprobes.



**Fig. 1 – 2** Mosaic symptoms on cherry leaves from trees infected with ASSVd.



**Fig. 3** White spots on cherry fruit from a tree cv 'Tragana Edessis' infected with ASSVd.

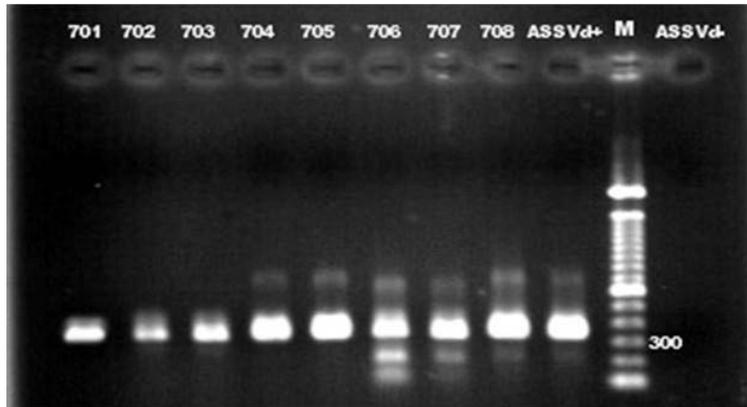
**Cloning and sequencing:** RT-PCR products of approximately 330 nt were either directly sequenced or cloned into pGEM-T and pCR® II plasmid vectors, according to the pGEM-T Easy (Promega, Madison, WI, USA) and TOPO-TA (Invitrogen, Carlsbad, CA, USA) cloning kit instructions, and then sequenced. The sequences obtained were compared with others in the NCBI database and those identified as complete sequence viroid genomes were submitted to the GenBank.

**Transmissibility tests:** Eight cherry rootstocks were bud-grafted with cherry tree buds from the orchard in September 2008 and tested by imprint hybridization and RT-PCR in May 2009.

## Results

**RT-PCR:** The originally tested sweet cherry sample with ASSVd specific primers gave an amplicon of about 330 nt. This amplicon was directly sequenced with both primers of the reaction and found to be 96-97% homologous to ASSVd. ASSVd-positive results were obtained for all 6 sweet cherry trees of the orchard examined (Florina, Macedonia). Neighboring apple samples were not found to be infected by ASSVd or other viroids, using RT-PCR, PAGE and Northern hybridization.

Transmissibility tests: Grafted rootstocks were found positive when tested with ASSVd primers in RT-PCR (Fig.4) and tissue-print hybridization.



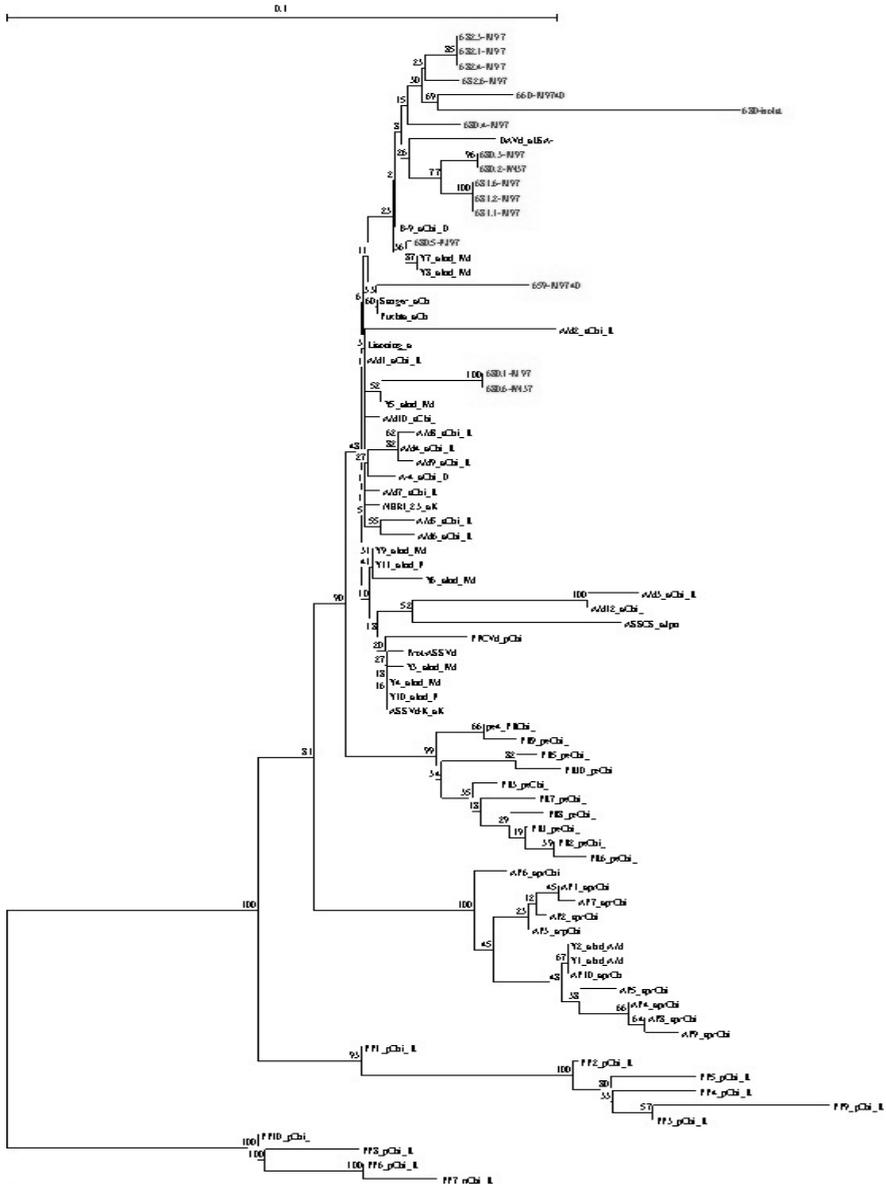
**Fig. 4** ASSVd positive RT-PCR products from 8 bud-grafted cherry rootstocks with ASSVd-positive material. From left to right: Lanes 1-8 rootstock samples, lane 9 positive control; lane 10, marker 100 bps (Invitrogen, UK), lane 11 negative control.

Cloning and sequencing: ASSVd-positive results from 5 cherry trees were initially confirmed by direct sequencing, and then by cloning and sequencing, resulting in a total number of about 20 complete ASSVd variants. From these, 16 were deposited in the GenBank under the accession numbers FJ974062-FJ974074, FN376408-FN396409 and GQ249350.

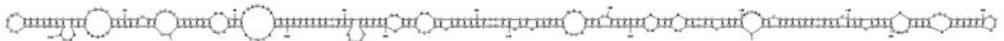
Sequence analysis: Hellenic ASSVd sequences from sweet cherry from clones and directly-sequenced RT-PCR products are 327-340 nt long. They differ from the prototype isolates of ASSVd (ASSCS and Y00435, Hashimoto and Koganezawa 1987) at 4-29 sites. There are 15 nucleotide changes between ASSCS and all Hellenic ASSVd cherry variants. The ASSVd sequences from Hellenic cherry share great homology with all Asian (Indian and Chinese) ASSVd sequences from apple recovered from the NCBI GenBank (93-99% or difference at 1-20 nt) (Fig.5).

In particular, their similarity to the Indian isolates from apple, Y5, Y7 and Y8, is 96-99% (difference at 1-13 nt). They also share a varying homology (91-98%) with Hellenic ASSVd variants from pome fruit trees (difference at 1-30 nt), whereas the heterogeneity among themselves fluctuates between 0 and 10% (0-34 nt).

The secondary structure of cherry ASSVd variants is rod-like (Fig.6).



**Fig. 5** Phylogenetic tree (neighbour joining analysis) of ASSVd containing the 16 Hellenic cherry sequences (red). Hellenic ASSVd cherry sequences do not form a separate cluster.



**Fig. 6** Secondary structure of the ASSVd clone sequence 680.5 from sweet cherry (MFold).

## Discussion

There are 15 nucleotide changes (differences from ASSCS) common to all Hellenic ASSVd variants, including cherry and pome fruit tree variants. There are no cherry-specific nucleotide changes in the ASSVd sequences obtained, therefore these sequences do not form a separate cluster in phylogenetic trees (Fig.5). The sequence variation among sweet cherry ASSVd variants is significant (10%), whereas the overall difference between all cherry sequences and other ASSVd variants does not exceed 10%. The fact that cherry trees harboring ASSVd sequences showed symptoms such as mosaic needs to be examined as to their cause-effect relation. To our knowledge, this is the first published report of detecting ASSVd in naturally infected cherry, including its molecular analysis. Northern blot hybridization analysis is under way in order to have a definitive proof of ASSVd presence in cherry.

## Acknowledgements

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