

## Molecular characterization of Hellenic variants of *Apple scar skin viroid* and *Pear blister canker viroid* in pome fruit trees

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### Abstract

*Apple scar skin viroid* (ASSVd) and *Pear blister canker viroid* (PBCVd) are members of the genus *Apscaviroid* (family *Pospiviroidae*). In order to study the nucleotide sequence and secondary structure of Hellenic variants of these viroids, a large number of collected samples were initially screened by imprint hybridization; then ASSVd and PBCVd positive samples were assayed for the viroids by RT-PCR. Total RNA extracts were reverse-transcribed and amplified by polymerase chain reaction using two different specific primer pairs for each viroid. Purified RT-PCR products were directly sequenced or cloned into the pGEM-T and pCR® II vectors and then sequenced. Fourteen Hellenic full length ASSVd variants from 3 apple, 3 wild apple (*Malus sylvestris*), 1 wild pear (*Pyrus amygdaliformis*) and 3 pear trees are 330-335 nucleotides long. They differ from the reference sequences of ASSVd (ASSCS and Y00435) at 15-29 and 3-36 sites, respectively. Fifteen nucleotide changes (differences from ASSCS) are common among all Hellenic variants. Hellenic ASSVd variants share high identity (97-100%) with ASSVd isolates from Asian apples. Three Hellenic variants, deriving from different hosts and areas, are identical with each other (wild apple and apple from Pella [Macedonia] and pear from Achaia [Peloponnesus]) and with another group of 3 apple variants from China (Liaoning, AM1 and B-9). Sixteen full length Hellenic PBCVd variants from 12 trees (4 apples, 1 wild apple, 5 pears, 1 wild pear and 1 quince) are 314-316 nucleotides long. There are 6-50 nucleotide changes among all Hellenic variants and the prototype PBCVd isolate (NC001830). Twenty-two (22) changes are identical among the majority of the Hellenic variants, regardless of origin, and 28-35 changes occur in PBCVd sequences obtained from apple and wild apple samples. In addition, 2 Hellenic PBCVd variants are 97-98% homologous to some Australian and European (Bosnian) PBCVd pear isolates, whereas the remaining 14 share 86-94% identity with Australian PBCVd isolates from pear, quince and Japanese pear (*Pyrus pyrifolia*). This is the first detailed molecular study of ASSVd and PBCVd in Hellenic cultivated and wild pome fruit trees.

Keywords: ASSVd, PBCVd, pome fruit, molecular characterization

### Introduction

*Apple scar skin viroid* (ASSVd) and *Pear blister canker viroid* (PBCVd) are members of the genus *Apscaviroid* (family *Pospiviroidae*) (Flores et al., 2003b). ASSVd variants are 329-334 nt long (Koganezawa et al. 2003; Kyriakopoulou et al. 2003) and PBCVd variants are 315-316 nt long (Flores et al. 2003a). Both induce serious diseases on pome fruit trees, such as apple scar skin, dapple apple, pear rusty fruit, pear dimple fruit (ASSVd) and pear blister canker (PBCVd) (Koganezawa et al. 2003; Kyriakopoulou et al. 2003; Flores et al. 2003).



**Fig. 1** ASSVd symptoms on pear cv. 'Kontoula' (Argolis, Peloponnesus)



**Fig. 2** PBCVd symptoms on pear cv. 'Kontoula' (Argolis, Peloponnesus)

In Greece, ASSVd and PBCVd have been reported to induce russetting, scarring and cracking on fruit (ASSVd) and blister canker on branches and twigs (PBCVd) of pear (*Pyrus communis*) (Fig. 1-2) and wild pear (*Pyrus amygdaliformis*) (Kyriakopoulou and Hadidi 1998; Kyriakopoulou et al. 2001), whereas ASSVd has also been detected on apple (*Malus domestica* Borkh) and wild apple (*Malus sylvestris*) trees (Boubourakas et al. 2008).

## Materials and methods

During 2006-2009, 772 wild and cultivated pome fruit tree samples with various symptoms were collected in the regions of Macedonia, Peloponnesus, Thessaly and Attica. The samples were initially examined by imprint hybridization, using a modified protocol of Palacio-Bielsa et al. (1999) and full-length DIG-labelled probes synthesized in Greece and in Italy by RT-PCR and *in vitro* transcription. In addition, total RNA phenol extracts of 54 samples (8 wild pear, 3 wild apple, 21 pear, 17 apple, 5 quince) were used in a one tube-two step RT-PCR protocol, employing two different primer sets per viroid (Table 1), as described by Faggioli et al. (2001). RT-PCR products of the expected size were either sequenced directly 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.

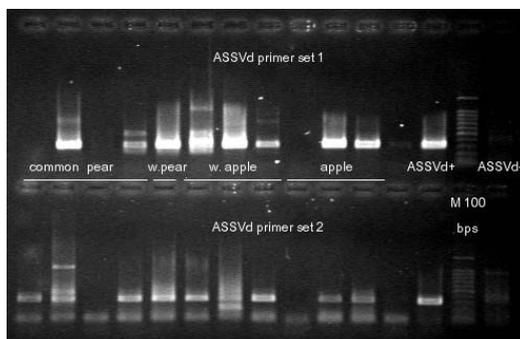
**Tab. 1** Primers used for the detection of ASSVd and PBCVd

Primer name	Sequence	Position	Amplicon (nts)
ASSVd1-H <sup>a</sup>	5'-CCGGTGAGAAAGGAGCTGCCAGCAC-3'	98-122	~330
ASSVd1-C <sup>a</sup>	5'-CCTTCGTCGACGACGA-3'	82-97	
ASSVdDSH <sup>b</sup>	5'-CGGTGACAAAGGAGCTGCCAG-3'	98-118	~330
ASSVdDSC <sup>b</sup>	5'-GCCTCCGTCGACGACGACAG-3'	83-102	
PBCVd1-H <sup>c</sup>	5'-TTGCTTGCCCTGAGCCTCGTCTTC-3'	167-202	~315
PBCVd1-C <sup>c</sup>	5'-CGCTGGTTTTCTCCAAAGGAGCGATTACTCACA-3'	131-167	
PBCVd3-H <sup>d</sup>	5'-GGAGCGCGCGGCTGTGAGTAATC-3'	121-143	~315
PBCVd3-C <sup>d</sup>	5'-AGCCAGCGCCAGGCTTCTAGACCCTT-3'	93-120	
PBCVd4-H <sup>e</sup>	5'-GTCTAGAAGCTGGGCGCTGGCTGG-3'	97-121	~315
PBCVd4-C <sup>e</sup>	5'-CCTTCGTCGACGACGA-3'	80-95	

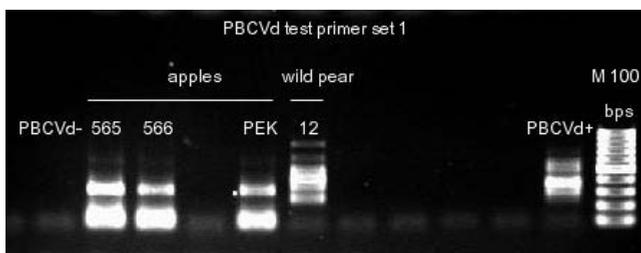
<sup>a</sup>Hadidi and Yang 1990; <sup>b</sup>Di Serio et al. 2002; <sup>c</sup>Loreti et al. 1997; <sup>d</sup>Shamloul et al. 2002; <sup>e</sup>F. Faggioli

## Results and discussion

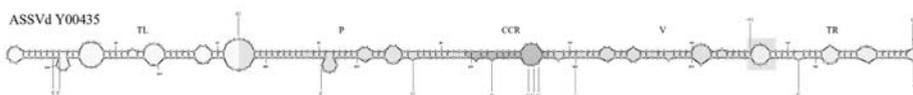
RT-PCR detected ASSVd infection in 27 pome fruit samples (9 pear, 6 wild pear, 1 quince, 3 wild apple, 8 apple) (Fig. 3) and PBCVd infection in 23 (8 pear, 4 wild pear, 1 quince, 3 wild apple, 7 apple) (Fig. 4). Cloning and sequencing resulted in 44 ASSVd and 21 PBCVd sequences. Complete sequences obtained from 14 ASSVd variants (4 apple, 3 wild apple, 5 pear, 2 wild pear) and 16 PBCVd variants (7 apple, 2 wild apple, 5 pear, 1 wild pear, 1 quince) were deposited in the GenBank under the accession numbers FJ974082-FJ974104, EU978462-EU978464, GQ141739-GQ141740, GQ249347 and GQ249349.



**Fig. 3** RT-PCR test on 4 pear, 1 wild pear, 3 wild apple and 3 apple phenol extracts using 2 ASSVd primer sets. From left to right: Lanes 1-11, pome fruit samples; lane 12, cherry sample; lane 13, positive controls; lane 14, marker 100 bps (Fermentas, LTU), lane 15, healthy controls.



**Fig. 4** PBCVd-positive RT-PCR products from apple and wild pear phenol extracts. From left to right: Lane 1, healthy control; lanes 2-9, pome fruit samples; lane 10, positive control; lane 11, marker 100 bps (Fermentas, LTU).



**Fig. 5** Secondary structure of the ASSVd Y00435 reference sequence (Hashimoto and Koganezawa 1987). The lines indicate common nucleotide differences of all Hellenic variants from ASSCS (black) and Y00435 (red) reference sequences.

The 14 complete Hellenic ASSVd sequences, 330-335 nucleotides long, differ from the ASSVd reference sequences (ASSCS and Y00435, Hashimoto and Koganezawa 1987) by 15-29 and 3-36 nts, respectively. Considering reference sequence ASSCS, 15 nucleotide changes are identical among all Hellenic variants, spread throughout all regions sharing a very high identity (97-100%) with Asian ASSVd sequences from apple (Indian, Chinese, Korean) (Fig.7).

The variation among themselves is 16% equaling that of the Chinese ASSVd sequences. Three Hellenic variants, from apple (Pella, Macedonia), wild apple (Pella) and pear (Achaia, Peloponnesus), were found to be identical to each other and to another group of 3 apple variants from China (Liaoning, AM1 and B-9) (Fig. 6). The 16 complete Hellenic PBCVd variants are 314-316 nt long. Fourteen of them have an identity of 86-94% with the Australian PBCVd sequences from pear, Japanese pear and quince, and a higher identity (97-98%) with European (mainly Bosnian) and other Australian PBCVd sequences from pear. Hellenic PBCVd sequences show significant variation among themselves (16%), double the variation among all the other known PBCVd sequences (8%) (Fig.8).

The 16 Hellenic variants differ from the reference sequence of PBCVd (Hernandez et al. 1992) at 6-50 positions. Four of these differences (gap-1C, gap-50A, G235C and gap-236U) are common for all Hellenic variants, whereas 22 of them are identical among the majority of the Hellenic variants, regardless of origin. The differences shown by the Hellenic apple and wild apple variants are 28-35 nt, compared to the reference sequence. The Hellenic PBCVd sequences have quasi-linear secondary structures (data not shown).

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AFCVd_AM1      GGTAAACACCGTGC GGTTCCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
ASSVd          GGTAAACACCGTGC GGTTCCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
Liaoning      GGTAAACACCGTGC GGTTCCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
334-FJ974097  GGTAAACACCGTGC GGTTCCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
392-FJ974099  GGTAAACACCGTGC GGTTCCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
565-FJ974098  GGTAAACACCGTGC GGTTCCCTGTGGTTCGCCCCGCCAACGCAGATAGATAAAGAAAACGA
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AFCVd_AM1      GGAGAAGAAGGAAC TCACTGTCGTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
ASSVd          GGAGAAGAAGGAAC TCACTGTCGTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
Liaoning      GGAGAAGAAGGAAC TCACTGTCGTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
334-FJ974097  GGAGAAGAAGGAAC TCACTGTCGTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
392-FJ974099  GGAGAAGAAGGAAC TCACTGTCGTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
565-FJ974098  GGAGAAGAAGGAAC TCACTGTCGTGTCGACGAAGGCCGGTGAGAAAGGAGCTGCCAGC
*****

AFCVd_AM1      ACTAAGCCGGACGG CGCCCTCGCACCAGTTC CGCTGTGGGTTTCGCCTACAAGAACGTACG
ASSVd          ACTAAGCCGGACGG CGCCCTCGCACCAGTTC CGCTGTGGGTTTCGCCTACAAGAACGTACG
Liaoning      ACTAAGCCGGACGG CGCCCTCGCACCAGTTC CGCTGTGGGTTTCGCCTACAAGAACGTACG
334-FJ974097  ACTAAGCCGGACGG CGCCCTCGCACCAGTTC CGCTGTGGGTTTCGCCTACAAGAACGTACG
392-FJ974099  ACTAAGCCGGACGG CGCCCTCGCACCAGTTC CGCTGTGGGTTTCGCCTACAAGAACGTACG
565-FJ974098  ACTAAGCCGGACGG CGCCCTCGCACCAGTTC CGCTGTGGGTTTCGCCTACAAGAACGTACG
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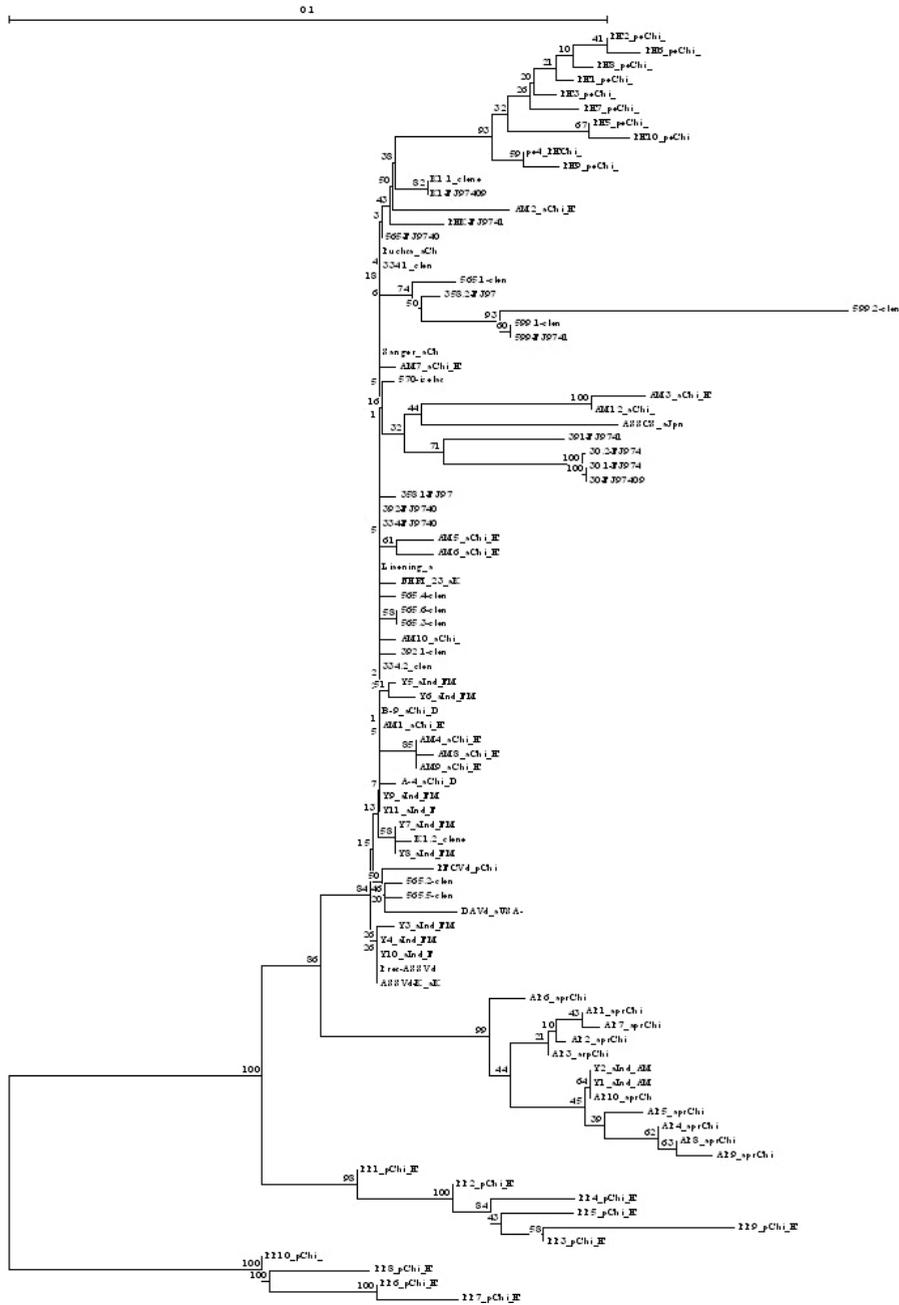
AFCVd_AM1      GTGTTGAGGCCCTG TCCGCCGCTGC GCTGCCACCTACTCTCGCGCCGCTAGTTCGAGCGGA
ASSVd          GTGTTGAGGCCCTG TCCGCCGCTGC GCTGCCACCTACTCTCGCGCCGCTAGTTCGAGCGGA
Liaoning      GTGTTGAGGCCCTG TCCGCCGCTGC GCTGCCACCTACTCTCGCGCCGCTAGTTCGAGCGGA
334-FJ974097  GTGTTGAGGCCCTG TCCGCCGCTGC GCTGCCACCTACTCTCGCGCCGCTAGTTCGAGCGGA
392-FJ974099  GTGTTGAGGCCCTG TCCGCCGCTGC GCTGCCACCTACTCTCGCGCCGCTAGTTCGAGCGGA
565-FJ974098  GTGTTGAGGCCCTG TCCGCCGCTGC GCTGCCACCTACTCTCGCGCCGCTAGTTCGAGCGGA
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AFCVd_AM1      CTCCGGGTGGAGCC CCGCTGTTCTCTC ACGCTCTTTTCTTTGACGCAGCGCGGGTGGGT
ASSVd          CTCCGGGTGGAGCC CCGCTGTTCTCTC ACGCTCTTTTCTTTGACGCAGCGCGGGTGGGT
Liaoning      CTCCGGGTGGAGCC CCGCTGTTCTCTC ACGCTCTTTTCTTTGACGCAGCGCGGGTGGGT
334-FJ974097  CTCCGGGTGGAGCC CCGCTGTTCTCTC ACGCTCTTTTCTTTGACGCAGCGCGGGTGGGT
392-FJ974099  CTCCGGGTGGAGCC CCGCTGTTCTCTC ACGCTCTTTTCTTTGACGCAGCGCGGGTGGGT
565-FJ974098  CTCCGGGTGGAGCC CCGCTGTTCTCTC ACGCTCTTTTCTTTGACGCAGCGCGGGTGGGT
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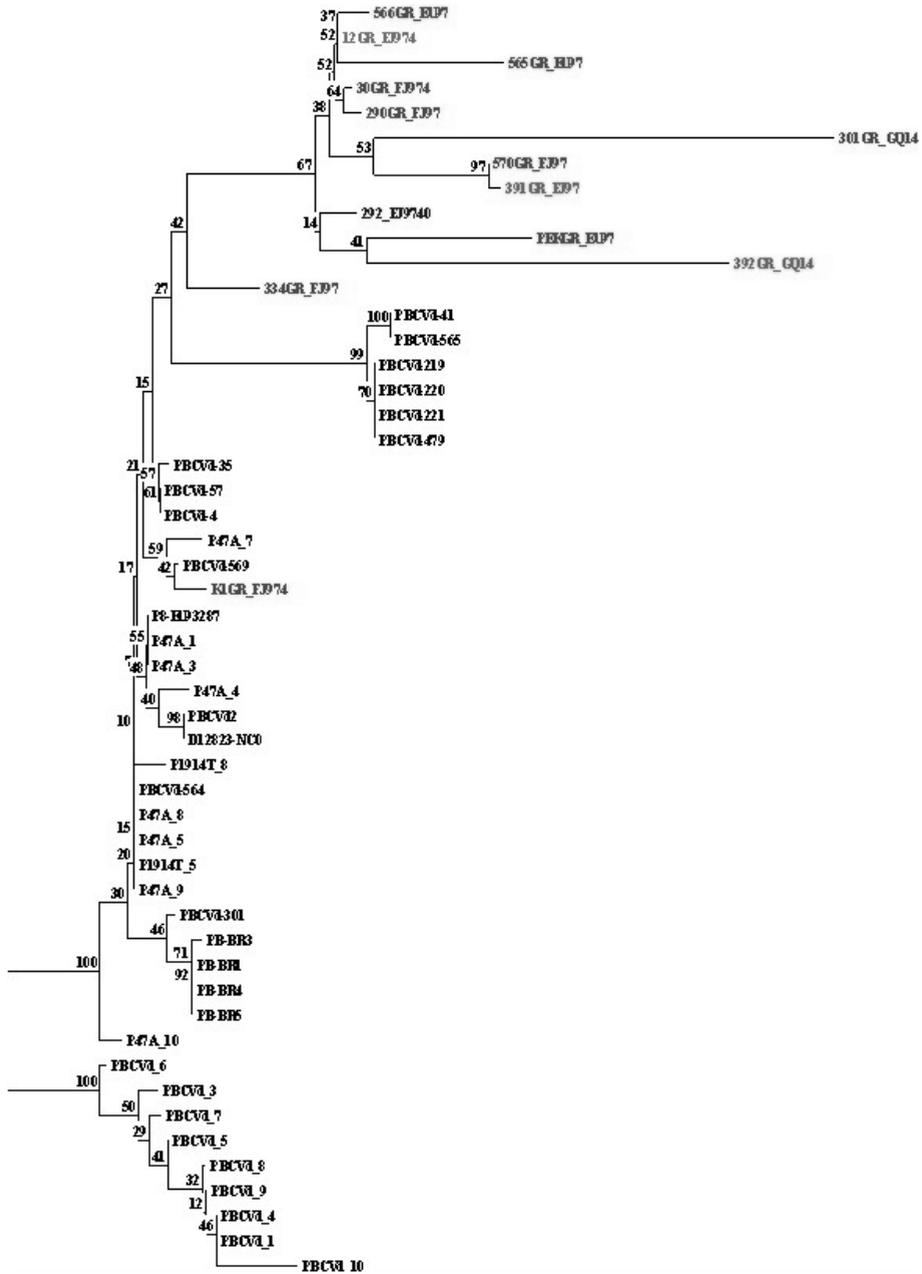
AFCVd_AM1      TCCCAGGGTAAAA CACAATAGGTGTTTCCC
ASSVd          TCCCAGGGTAAAA CACAATAGGTGTTTCCC
Liaoning      TCCCAGGGTAAAA CACAATAGGTGTTTCCC
334-FJ974097  TCCCAGGGTAAAA CACAATAGGTGTTTCCC
392-FJ974099  TCCCAGGGTAAAA CACAATAGGTGTTTCCC
565-FJ974098  TCCCAGGGTAAAA CACAATAGGTGTTTCCC
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**Fig. 6** Alignment of 3 Chinese (apple) and 3 Hellenic (334-pear, 392-wild apple, 565-apple) ASSVd sequences with CLUSTAL.



**Fig. 7** Phylogenetic diagram of 26 Hellenic ASSVd pome fruit sequences (blue) and other 63 pome fruit sequences (neighbour-joining analysis, bootstrap=100)



**Fig. 8** Phylogenetic diagram of 13 Hellenic PBCVd pome fruit sequences (colored) and other 38 pome fruit sequences (neighbour-joining analysis, bootstrap=100).

This is the first detailed molecular study of ASSVd and PBCVd in Hellenic pome fruit orchards and wild pome fruit trees. The wide host range of ASSVd and PBCVd in Greece includes 5 pome fruit species, 2 of which are wild species of pear and apple, which grow in mountains far away from cultivated crops. The widespread occurrence of these two viroids in local pome fruit varieties as well as in wild species, and the wide variation of their Hellenic sequences, are indications that these viroids are probably native to Greece.

## Acknowledgements

This research project is co-financed by E.U.-European Social Fund (75%) and the Greek Ministry of Development-GSRT (25%).

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