

## INCIDENCE AND MOLECULAR VARIABILITY OF *APPLE STEM PITTING* AND *APPLE CHLOROTIC LEAF SPOT* VIRUSES IN APPLE AND PEAR ORCHARDS IN GREECE

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### SUMMARY

A survey was conducted in the major pome fruit growing districts of Greece to assess the incidence of *Apple stem pitting virus* (ASPV) and *Apple chlorotic leaf spot virus* (ACLSV) using RT-PCR assays amplifying part of the RNA-dependent RNA polymerase (RdRp) and the coat protein genes of the viruses, respectively. ASPV was predominant in apple (91.8%) and pear (51.3%) and was detected in almost all the cultivars and rootstocks tested. It was also found for the first time in the Japanese pear (*Pyrus serotina*). The incidence of ACLSV in apples was also high (65.7%) and mixed infections with both viruses were frequently encountered. In pear, ACLSV incidence was lower (20.7%). This is the first extensive survey conducted in Greece for monitoring ACLSV and ASPV using molecular assays. Nucleotide sequencing of different apple and pear ASPV and ACLSV isolates and comparative analysis with already published homologous genomic regions revealed a significant level of variation up to 29% and 22% for the RdRp and the CP genes, respectively. However, high identity rates were found among isolates of both viruses from different geographical origins. Our results show the high impact of the use of infected plant material in the dissemination of ASPV and ACLSV and illustrate the need to implement programs for the production of certified pome fruit propagating material in Greece, in order to prevent further spread of the viruses.

*Key words:* ASPV, ACLSV, survey, molecular variability, RT-PCR, *Malus*, *Pyrus*.

### INTRODUCTION

Apple and pear are very important crops in Greece where they are mainly cultivated in the centre and north of the country. *Apple stem pitting virus* (ASPV) and *Apple chlorotic leaf spot virus* (ACLSV), members of the

family *Flexiviridae* (Adams *et al.*, 2004), are among the most important viruses of apple and pear worldwide (Nemeth, 1986). Although frequently latent, they are responsible for serious diseases such as epinasty and decline of Spy 227 (Stouffer, 1989), quince fruit deformation (Mathioudakis *et al.*, 2009), pear vein yellows (Leone *et al.*, 1998), plum pseudopox, russet ring and bitterrot (Desvignes and Boye, 1988). No vector is currently known and it is likely that virus dissemination is due to the use of infected propagating material. Thus, the development and application of reliable certification schemes are necessary for their control.

In Greece, ACLSV was first reported in apples in 1964 (Anonymous, 1971) and recently in quince (Mathioudakis *et al.*, 2007) but data concerning its incidence on apple and pear are limited (Varveri and Bem, 1995). On the other hand, ASPV was initially identified in a small number of apple trees by graft inoculation onto indicator plants (Syrgianidis, 1988) and recently by using molecular assays in quince, apple and pear (Mathioudakis *et al.*, 2006). No information exists on ASPV incidence in apples and pears.

Previous studies have shown a high molecular variability in the coat protein (CP) gene of these viruses (Candresse *et al.*, 1995; Nemchinov *et al.*, 1998; Schwarz and Jelkmann, 1998; Yoshikawa *et al.*, 2001; Al Rwahnih *et al.*, 2004). Furthermore, a 10-21% divergence has been reported in the RdRp gene of a small number of ASPV isolates from Poland and Greece (Malinowski *et al.*, 1998; Mathioudakis *et al.*, 2009) compared to the available sequences in the GenBank database.

The present study was undertaken to provide a further insight on the distribution and relative importance of ASPV and ACLSV on apple and pear orchards in Greece. In addition, new partial RdRp and CP sequences were determined from various ASPV and ACLSV isolates, from different geographical regions and were used, along with published homologous genomic regions, to produce a thorough molecular variability analysis.

### MATERIALS AND METHODS

**Survey for ASPV and ACLSV.** A survey was carried

out from March 2005 to October 2006 in 19 geographical districts of Greece; 245 samples, 134 from apples and 111 from pears, were randomly collected (Table 1). The greatest number of samples originated from the key pome fruit growing areas including Imathia, Magnisia and Larissa (Table 1). With the exception of typical mosaic symptoms caused by *Apple mosaic virus* (ApMV) present in apple trees (data not shown), no symptoms of virus infection were observed in the orchards surveyed. Each sample consisted of mixed plant tissues which included bark, leaves and petioles. For template preparation, spotting of plant sap onto nylon membranes, and total RNA extraction were applied for ASPV and ACLSV, respectively (Mathioudakis *et al.*, 2009).

**Virus detection.** ASPV was detected by a spot nested RT-PCR, using degenerate primers with a broad detection range which amplify a 312 bp part of the RdRp gene (Mathioudakis *et al.*, 2009). For ACLSV an already well-established one tube RT-PCR was applied using the primers A52 (5'- CAGACCCTTATTGAAGTCGTCGAA-3') and A53 (5'- GGCAACCCTGGAACAGA-3'), which amplify a 358 bp fragment of the CP gene (Candresse *et al.*, 1995). For confirmation, a new pair of primers (CLSup: 5'- CTGGAACAGATACTGGAGTC-3'; CLSdo: 5'- GCCTTGTTTCATGATRAACAT-3') were designed on conserved regions of the CP gene, after aligning homologous nucleotide sequences from different ACLSV isolates (accession Nos. AJ243438, M58152, X99752, D14996, AB060960, AY669389, AF251275, AJ586643) including three more trichoviruses (*Apricot pseudo-chlorotic leaf spot virus*, AY713379; *Cherry mottle leaf virus*, AF170028; *Grapevine berry inner necrosis virus*, D88448), using the CLUSTAL-X package (Thompson *et al.*, 1997).

The primers, which amplify a 374 bp fragment, were used in a one-tube RT-PCR (25 µl) in a final concentration of 0.2 µM (CLSup) and 0.4 µM (CLSdo) along with 2 µl RNA extract, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5% DMSO, 0.5 mM DTT, 0.25 mM dNTPs, 12 units RNASEOUT (Invitrogen, The Netherlands), 0.8 units Superscript<sup>TM</sup> II RNase H Reverse Transcriptase (RT) (Invitrogen, The Netherlands), 0.8 units AMV RT (Finnzymes, Finland) and 1 unit Dynazyme II<sup>TM</sup> DNA polymerase (Finnzymes, Finland). The cycling scheme was as follows: 47°C for 50 min, 50°C for 7 min, 94°C for 4 min, 40 cycles of 94°C for 30 sec, 51°C for 30 sec, 72°C for 30 sec and a final elongation step at 72°C for 2 min.

**Sequence analysis.** PCR products were purified with a matrix gel extraction system (Marligen Bioscience, USA) and directly sequenced using the virus-specific primers. The nucleotide sequences of 6 amplicons from different apple and pear isolates were determined for each virus (Table 2). The sequences obtained were com-

pared, after clipping the primer-binding regions, with those of the homologous genes of published ASPV and ACLSV isolates using the BLAST program (Altschul *et al.*, 1990), while multiple nucleotide and amino acid alignments were made using the CLUSTAL-X alignment routine (Thompson *et al.*, 1997).

## RESULTS

**Incidence of ASPV and ACLSV.** At least one virus was present in 185 out of 245 samples tested (Table 1), ASPV being the most common. It was detected in almost all apple trees tested, with a rate of 91.8% (123/134) and was also present in 51.3% (57/111) of the pear samples. ACLSV was detected in 105 samples using the A52/A53 primer pair. However, the one-tube RT-PCR developed in this study detected the virus in six more samples (two apples from Chalkidiki, one apple from Larissa and two pears from Florina and Imathia, respectively). Overall, ACLSV incidence in apple trees was 65.7% (88/134) but only 20.7% (23/111) in pears. Mixed infections with the two viruses were also found in 85 apple (63.4%) and 20 pear (18.0%) samples (Table 1). Interestingly, ASPV was present in the 96.6% of the ACLSV infected-apple trees.

ACLSV was not detected in apple cvs Delicious Pifafa, Golden Sweet, Schniga and Pink Lady nor in the two rootstocks EM 9 and MM 106. However, the number of samples tested was very small and therefore large-scale surveys are needed in order to further clarify whether they are actually infected with ACLSV. On the other hand, only Coscia, Highland, Kristalli, Tsakoniki and some undetermined pear cultivars were infected by ACLSV. ASPV was present in all the apple and pear cultivars as well as the two rootstocks tested. Moreover, ASPV was isolated from two naturally infected cultivars (Kosui, Hosui) of the Japanese pear *P. serotina*, and the pear rootstocks Old Home x Fragmindale (OH x F), contrary to ACLSV (Table 1).

**Molecular variability of the partial RdRp gene of ASPV.** The sequences of ASPV nested PCR products from six isolates from different geographic regions (Table 2) were determined and compared with those from five isolates available in the GeneBank, reported from Greece, Germany, China and Japan. The overall results of the analysis showed a significant range of divergence from 6 to 29% and 0 to 27% at the nucleotide and amino acid level, respectively, which was evenly distributed along the partial RdRp sequence except for the ASPV-EM9 isolate which displayed a significant divergence at aa positions 40-48 (Table 3, Fig. 1A). In fact, ASPV-EM9 was the most distantly related isolate showing only 71% and 73% nucleotide and amino acid identities with the PR1 isolate (China) (Table 3, Fig. 1A).

**Table 1.** Incidence of *Apple stem pitting virus* and *Apple chlorotic leaf spot virus* in different areas and apple and pear cultivars.

Geographic area	Species and cultivar		No of samples collected		ACLSV positive		ASPV positive		Mixed infections	
	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear
Arkadia	Delicious Pilafa	Abate Fetel	2	1	0	0	2	1		
	Gala	Kristalli	2	2	2	2	2	2	3	2
	Golden Sweet	Passa Crassana	2	2	0	0	2	0		
	Red Chief	-	2	-	1	-	2	-		
Arta	-	Unknown	-	1	-	0	-	0	-	0
Chalkidiki	Unknown	Kontoula		1		0		0		
		Kristalli	5	1	2	0	4	1	2	0
		Tsakoniki		1		0		0		
		Unknown		5		0		2		
Chania	Unknown	-	1	-	0	-	1	-	0	-
Florina	Black Ben Davis	Highland	1	1	1	0	1	0		
		Kontoula		1		0		0	2	1
		Kristalli	1	4	1	2	1	2		
		Williams		1		0		1		
Fthiotida	Granny Smith	Kontoula	1	2	0	0	1	0	1	0
	Starking		1		1		1			
Grevena	Unknown	Unknown	4	6	2	1	2	1	2	1
Imathia	Golden Sweet	Etrusca	12	1	11	0	12	1		
	Granny Smith	Highland	1	2	1	1	1	1		
	Pink Lady	Kontoula	1	2	0	0	1	2		
	Red Chief	Kristalli	13	4	11	2	13	3	25	3
	Royal Gala	Tsakoniki	1	1	1	1	1	1		
	Scarlet Spur	Santa Maria	1	1	1	0	1	1		
Ioannina	Unknown	Unknown	16	5	13	5	13	5	12	5
	Firiki		1		1		1			
Karditsa	-	Unknown	-	1	-	0	-	0	-	0
Kavala	Unknown	-	1	-	1	-	1	-	1	-

Kozani	Firiki	Coscia	1	5	1	1	1	4	4	2
	Golden Delicious	Kontoula	7	3	1	0	7	2		
	Jonagold	Kristalli	1	2	1	1	1	2		
	Schniga	<i>Pyrus serotina</i> cv. Kosui	2	2	0	0	0	1		
Komotini	Starking	Unknown	1	1	1	1	1	1	1	1
Larissa	Firiki	Coscia		1		0		1		
		Harrod Sweet		1		0		1		
		Highland		4		0		1		
		Kontoula	8	2	6	0	8	0	6	0
		Kristalli		7		0		0		
		Unknown		4		1		1		
		Williams		6		0		4		
Magnisia	Imperial	Kristalli	1	1	1	0	1	1		
	Starking	Unknown	14	10	11	5	14	7	19	5
	Unknown	-	16	-	7	-	16	-		
Pella	Red Delicious	-	5	-	3	-	5	-	3	-
Rethymno	-	Kristalli	-	3	-	0	-	1	-	0
Thesprotia	-	Unknown	-	2	-	0	-	2	-	0
Thessaloniki	EM9 rootstock	Decana del Comisio	1	1	0	0	1	1		
	Firiki	Highland	2	1	2	0	2	0		
	MM106 rootstock	OH x F rootstock	1	3	0	0	1	1		
	Starking	Passa Crassana	1	1	0	0	1	1		
	Unknown	<i>P. serotina</i> cv. Hosui	4	1	2	0	2	1	4	0
	-	<i>P. serotina</i> cv. Kosui	-	1	-	0	-	0		
	-	<i>P. serotina</i> cv. Shinseiki	-	1	-	0	-	0		
	-	Santa Maria	-	1	-	0	-	0		
-	Unknown	-	1	-	0	-	0			
Total			134	111	88	23	123	57	85	20

:- no samples were collected

On the other hand, ASPV-KOZ shared 95% amino acid identity with the Japanese IF38 isolate. Among the Greek isolates the rate of variability ranged between 10 to 27% and 6 to 22% at the nucleotide and amino acid levels, respectively (Table 3). ASPV-MM106 and ASPV-EM9 were most distantly related to ASPV-GT11 (73 and 74% in nucleotides, respectively). Finally, ASPV-MM106 and ASPV-AFT5 sequences were identical (100%) at the amino acid level and 90% similar at the nucleotide level.

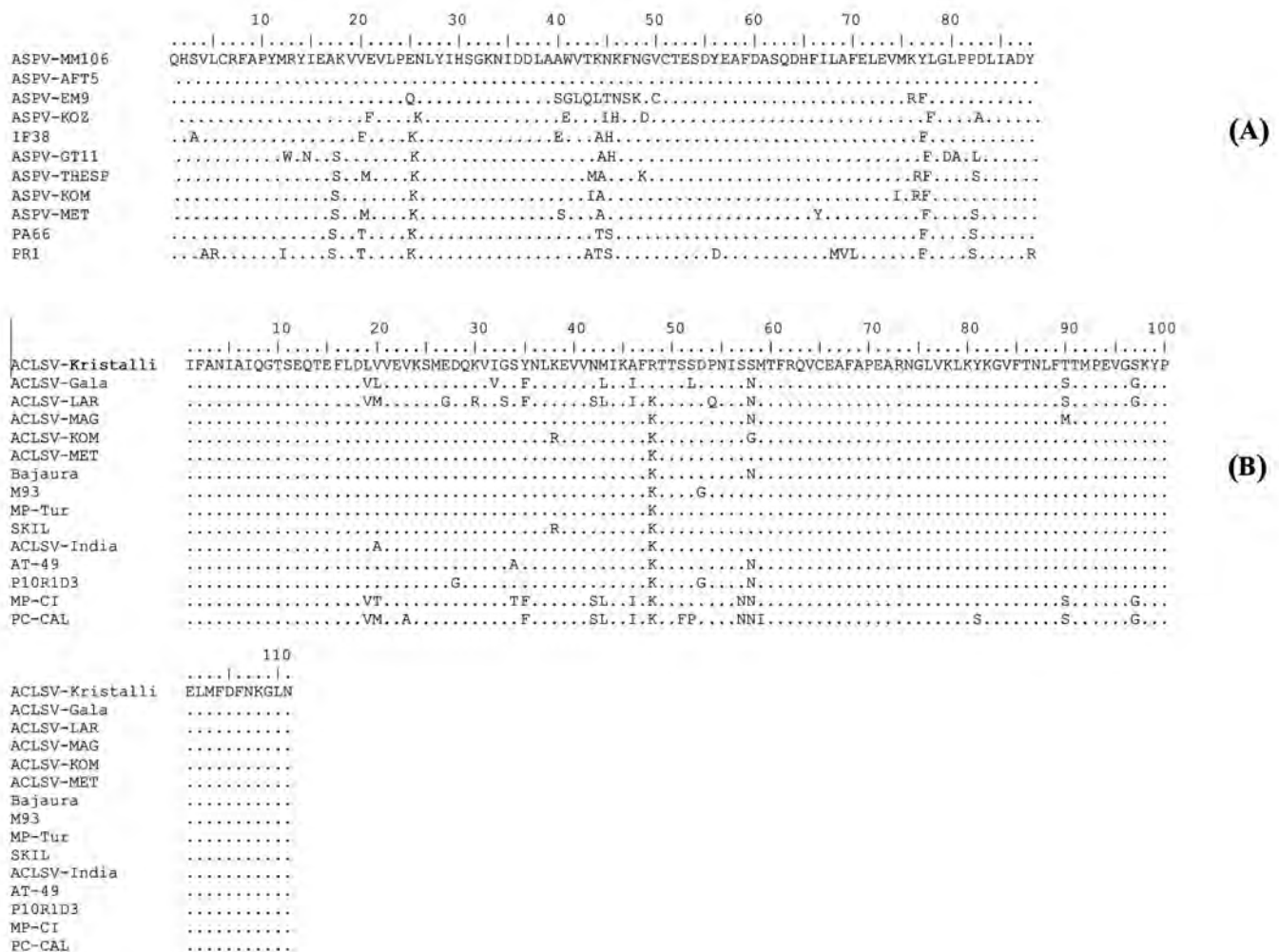
**Molecular variability of the partial CP gene of ACLSV.** Comparisons of the ACLSV sequences obtained with homologous published genomic regions confirmed the specificity of the one-tube RT-PCR assay developed herein. The nucleotide and amino acid variability of ACLSV was mainly encountered at the N-terminal half of the partial CP gene and ranged between 3 and 22% and 0 and 15%, respectively (Table 4, Fig. 1B). ACLSV-Kristalli was the most distantly related to the Italian PC-CAL isolate, displaying an identity of

81% and 79% at the amino acid and nucleotide levels, respectively. On the contrary, ACLSV-MAG was 99% identical at the aa level (97% at the nt level) to the Bajaura isolate from India and ACLSV-MET shared 100% identity with the Turkish MP-Tur isolate (93% at the nt level). Between the Greek isolates, ACLSV-LAR and ACLSV-MAG were the most distantly related (87% at the aa and 78% at the nt level, respectively), while ACLSV-MET was 98% identical at the aa level to ACLSV-MAG (94% at the nt level). Finally, ACLSV-MET shared a 99% aa identity with ACLSV-Kristalli (90% at the nt level) (Table 4).

All sequences determined in this study were deposited in the EMBL-EBI database under the accession numbers listed in Table 2.

**DISCUSSION**

ACLSV incidence was previously estimated in Greece by ELISA (Varveri and Bem, 1995), with a small



**Fig. 1.** Amino acid sequence alignments of the partial RdRp (A) and CP (B) genes from various ASPV and ACLSV isolates, respectively. The accession numbers of the sequences included are reported in Table 2.

**Table 2.** ASPV and ACLSV isolates used in this study and their EMBL Database accession numbers.

Isolate	Host/Cultivar	Origin	Accession no.
	Apple		
ASPV-MET*	Unknown	Greece/Ioannina	FN386781
ASPV-GT11	Golden Delicious	Greece/Thessaloniki	AM746059
IF38	nr	Japan	AB045371
ASPV-EM9*	Apple rootstock, East Malling 9	Greece/Thessaloniki	FM992635
ASPV-MM106*	Apple rootstock, Malling Merton 106	Greece/Thessaloniki	FM992636
PA66	Apple rootstock, East Malling 9	Germany	D21829
	Pear		
ASPV-KOZ*	Kristalli	Greece/Kozani	FN386782
ASPV-THESP*	Unknown	Greece/Thesprotia	FN386783
ASPV-KOM*	Unknown	Greece/Komotini	FN386784
ASPV-AFT5	Abate fetel	Greece/Thessaloniki	AM746060
PR1	nr	China	EU095327
	Apple		
ACLSV-Gala*	Gala	Greece/Arkadia	FM992638
ACLSV-MAG*	Starking	Greece/Magnisia	FN386785
ACLSV-MET*	Unknown	Greece/Ioannina	FN386786
Bajaura	nr	India	AM494510
M93	nr	Albania	AJ586626
MP-Tur	nr	Turkey	AJ586641
SKIL	nr	Israel	DQ834688
AT-49	nr	Hungary	AY677104
MP-CI	nr	China	AJ586640
P10R1D3	nr	Bulgaria	DQ329162
	Pear		
ACLSV-Kristalli*	Kristalli	Greece/Florina	FM992637
ACLSV-LAR*	Unknown	Greece/Larissa	FN386787
ACLSV-KOM*	Unknown	Greece/Komotini	FN386788
PC-CAL	nr	Italy	AJ586643
ACLSV-India	nr	India	AM882704

nr; not referred in database or in the Journal publication

\*; sequences determined in this study

**Table 3.** Nucleotide and amino acid sequence percentage (%) identities of the partial RdRp gene among 11 apple and pear isolates of *Apple stem pitting virus*. The lowest and highest percentage identities are indicated in bold.

aa \ nt	nt										
	ASPV-MET	ASPV-GT11	IF38	ASPV-EM9	ASPV-MM106	PA66	ASPV-KOZ	ASPV-THESP	ASPV-KOM	ASPV-AFT5	PR1
ASPV-MET		78	79	74	75	83	79	82	80	75	79
ASPV-GT11	88		78	74	73	78	78	79	79	73	74
IF38	92	88		73	79	80	82	78	79	78	76
ASPV-EM9	82	78	84		75	76	81	77	77	78	<b>71</b>
ASPV-MM106	90	87	92	85		80	78	75	76	90	75
PA66	94	89	92	82	92		76	85	87	77	<b>94</b>
ASPV-KOZ	90	87	95	84	90	92		76	76	77	<b>71</b>
ASPV-THESP	94	87	89	85	89	93	89		85	75	82
ASPV-KOM	92	88	90	85	92	92	88	94		74	83
ASPV-AFT5	90	87	92	85	<b>100</b>	92	90	89	92		73
PR1	84	80	81	<b>73</b>	81	89	81	84	82	81	

**Table 4.** Nucleotide and amino acid sequence percentage (%) identities of the partial CP gene among 15 apple and pear isolates of *Apple chlorotic leaf spot virus*. The lowest and highest percentage identities are indicated in bold.

nt aa	ACLSV- Gala	ACLSV- MAG	ACLSV- MET	Bajaure	M93	MP-Tur	SKIL	AT-49	MP-CI	P10RID3	ACLSV- Kistalli	ACLSV- LAR	ACLSV- KOM	PC- CAL	ACLSV- India
ACLSV-Gala		82	82	83	81	82	82	80	85	81	80	83	82	85	82
ACLSV-MAG	90		94	<b>97</b>	92	93	94	90	83	90	89	<b>78</b>	89	81	89
ACLSV-MET	90	98		93	92	93	92	90	83	90	90	79	89	82	89
Bajaure	90	99	99		92	94	95	91	84	90	90	80	89	82	90
M93	89	97	99	98		92	92	88	82	89	90	80	88	81	87
MP-Tur	90	98	<b>100</b>	99	99		94	91	83	92	90	80	89	82	88
SKIL	89	97	99	98	98	99		90	82	91	90	79	90	81	87
AT-49	89	97	97	98	96	97	96		85	90	86	80	86	81	85
MP-CI	93	90	90	90	89	90	89	90		84	81	87	81	89	84
P10RID3	89	97	97	98	98	97	96	96	89		89	79	87	81	87
ACLSV-Kistalli	90	97	99	98	98	96	98	96	89	96		81	87	79	87
ACLSV-LAR	91	89	88	89	87	88	87	87	93	87	87		<b>78</b>	86	80
ACLSV-KOM	89	97	98	98	97	98	99	96	89	96	97	87		81	85
PC-CAL	90	87	86	87	<b>85</b>	86	<b>85</b>	<b>85</b>	93	<b>85</b>	<b>85</b>	90	<b>85</b>	81	82
ACLSV-India	90	97	99	98	98	99	98	96	90	90	98	88	97	86	82

survey, whereas no information was gathered on the presence and distribution of ASPV. In this study a larger number of samples were collected from different areas for determining the frequency of these viruses and providing a relatively clear picture of the phytosanitary status of pome fruit crops in the country.

The results showed that ASPV and ACLSV are widespread in the Greek pome fruit industry. ASPV was predominant in both apple and pear cultivars and was distributed throughout the country. The virus was also detected in Nashi pears (*P. serotina*, Japanese pear) and to our knowledge this is the first report of ASPV in this species. Recently, Paunović and Jevremović (2004) recorded ASPV in another *Pyrus* species (*P. ussuriensis*) of Asian origin. We also detected ASPV in the two apple rootstocks tested, namely EM9 and MM106, as well as in the OH x F pear rootstocks. Likewise, ACLSV incidence was also high in apple trees and the virus was present in almost all the areas surveyed, but it was rather limited in pear trees. Similar results have been obtained by other researchers studying the incidence and spread of ASPV and ACLSV in other countries (Kundu, 2001, 2002, 2003; Aneliya, 2005; Salem *et al.*, 2005; Ulubas and Ertunc, 2005; Constable *et al.*, 2007).

The comparative sequence analysis made in this study confirmed the already known high molecular variability in the CP of ACLSV (Candresse *et al.*, 1995; Al Rwahnih *et al.*, 2004; Ulubas and Rosner, 2006) among various isolates. In fact this divergence, which was up to 22% in nucleotides and 15% in amino acids, was mainly encountered at the N-terminal half of the partial CP, as previously reported (Al Rwahnih *et al.*, 2004; Ulubas and Rosner, 2006).

In the case of ASPV sequence information on the RdRp gene is limited. Thus, our determination of the partial gene sequences from six different apple and pear isolates has enriched these data and allowed a more thorough analysis of the variability, in comparison with homologous published sequences. The results indicated high rates of divergence up to 29% at the nucleotide and 27% at the amino acid level in the case of the ACLSV-EM9 isolate (Fig. 1). The existence of a highly divergent amino acid motif lying between 40 and 50 aa site (Fig. 1) on this isolate might be due to a nucleotide deletion and insertion in nt sites 118 and 153 (data not shown). High divergence (76-96% identity) was also recorded in the 3' terminal end of the RdRp gene (Constable *et al.*, 2007).

Phylogenetic analysis was also implemented to investigate the evolutionary relationships among different isolates of the two viruses. However, no clear clustering according to host-species and geographic origin of the isolates was observed (data not shown). Actually, as revealed by the molecular variability analysis, isolates of both viruses from different geographical origins showed very high identity rates, indicating the prominent role of

exchanging infected plant material on virus dissemination.

The low sanitary status of apple and pear trees in Greece, regardless of the cultivar, calls for the implementation of certification schemes for the production of virus-free propagative material. A basic prerequisite for developing a certification scheme is the existence of a sensitive, reliable cost-effective and rapid detection method, suitable for large-scale application. So far the evaluation of the virus status of mother stocks and nurseries in Greece is based on the absence of characteristic symptoms. However, ASPV and ACLSV are frequently latent (Nemeth, 1986) and therefore this strategy favors their dissemination. Also, the common practice of grafting is an effective way to transmit viruses and may cause their wide distribution. In some cases, when infected scions are grafted on certain rootstocks, this can result in reduced production and tree decline (Kinard *et al.*, 1996; Desvignes, 1999).

The detection of ASPV and ACLSV by biological indexing on woody indicators, although sensitive, is time-consuming and involves handling difficulties and variation of symptoms, depending on the pathogenicity of the isolate and the indicator-host used (Stouffer and Fridlund, 1989; Paunovic and Jevremovic, 2004). Additionally, serological detection of ACLSV is mainly reliable in spring (Candresse *et al.*, 1995; Kinard *et al.*, 1996) and due to the virus variability the results are not consistent. Furthermore, monoclonal antibodies produced against ASPV (Gugerli and Ramel, 2004) did not give reliable results in some pear tissues tested by DAS-ELISA (Paunovic and Jevremovic, 2006). Therefore, the application of molecular assays, such as those reported herein is recommended for reliable screening of propagating material and prevention of virus spread.

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Received July 4, 2009

Accepted August 22, 2009

