GENETIC DIVERSITY OF FLEXIVIRUSES INFECTING POME FRUIT TREES

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SUMMARY

Three amplicons corresponding to the variable genome regions of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) were sequenced from different apple cultivars and geographic areas in Europe and Asia. Multiple alignments of nucleotide sequence of these isolates with those from databases showed a very high divergence. Genetic variability at the nucleotide level among ACSLV and ASPV isolates was very high, ranging from 83.5 to 85.0% and 80.1 to 81.9%, respectively, confirming previous observations. ASGV isolates were more homogenous, with no clear separation between ASGV and *Citrus tatter leaf virus* and between geographic origin and genetic diversity of the virus isolates characterized in this study.

Key words: ACLSV, ASPV, ASGV, phylogeny, molecular characterization.

INTRODUCTION

Pome fruits are hosts for many viruses, among which *Apple stem pitting virus* (ASPV, genus *Foveavirus*), *Apple stem grooving virus* (ASGV, genus *Capillovirus*), and *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*) all belonging in the family *Flexiviridae* (Adams *et al*., 2004). Most commercially grown apple and pear cultivars remain symptomless when infected by ASPV, ASGV and ACLSV. However, in susceptible apple, pear and quince cultivars the viruses are associated with economically important diseases such as apple stem pitting, apple epinasty and decline, pear vein yellows and red mottle, graft union necrosis, pear stony pit, quince sooty ring spot and fruit deformations (Leone *et al*., 1998; Desvignes *et al*., 1999). Several surveys have shown that these viruses are widely distributed in most of the pome fruit growing areas of Europe (Kundu, 2003a; Massart *et al*., 2008; Mathioudakis *et al*., 2006; Paunovic and Jevremovic, 2008), America, Asia, (Birisik *et al*., 2008; Dhir *et al*., 2009; Salem *et al*., 2005), Australia and New Zealand (Rodoni and Constable, 2008). The genetic variability of RNA viruses is high because of the lack of proofreading activity associated with RNA-dependent RNA polymerase so that single hosts may contain co-existing populations of diverse sequence variants (Yoshikawa *et al*., 2001).

The complete nucleotide sequences of the ASPV, ASGV and ACLSV genomes have been determined and sequence analyses have shown high variability. The most variable regions of the ACLSV genome are downstream of the methyl-transferase (MET) domain in the 216K ORF, the C-terminal part of the 50K movement protein ORF, and the N-terminal part of the 28K ORF, upstream of the coat protein (CP) coding region (German-Retana *et al*., 1997). One hypervariable region of the ASPV and ASGV genomes is located between the MET and P-Pro domains (Magome *et al*., 1999; Yoshikawa *et al*., 2001). The second variable genomic region of these viruses is located in the putative movement protein (MP) overlapping the N-terminal CP coding region in ASPV (Yoshikawa and Takahashi, 1988; Yoshikawa *et al*., 2001) and the V-region encoding ORF-2 in ASGV (Magome *et al*., 1999).

This paper presents an analysis of the genetic diversity of ASPV, ASGV and ACLSV from distinct geographical locations in Europe and Asia, i.e. Czech Republic, Republic of Serbia, Ukraine, Poland, Belgium, Turkey and India. The partial genome sequences of each selected isolate were deposited in GenBank database.

MATERIALS AND METHODS

Virus sources. The ACLSV, ASPV and ASGV isolates used in this study (Table 1) originated from apple

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Table 1. Incidence of ASPV, ASGV and ACLSV in apple trees surveyed in this study.

Countries	Total samples analyzed	Single infection ASPV	Single infection ASGV	Single infection ACLSV	Co-infection ASPV+ACLSV	Co-infection ASPV+ASGV	Co-infection ASGV+ACLSV	Co-infection ASPV+ACLSV+ASGV	Healthy plants
Czech Republic	32	2	5	0	0	16	0	6	3
Serbia	24	10	2	0	6	0	0	6	-
Ukraine	11	0	0	2	1	0	0	2	6
India	10	2	0	1	1	0	3	3	-
Poland	16	0	0	0	0	13	0	3	-
Belgium	16	0	0	2	6	2	2	4	-
Turkey	8	2	2	0	1	0	2	1	-

trees grown in Europe and Asia. Surveyed commercial orchards were established with uncertified planting material and infections were symptomless in most of them.

Isolation and virus detection by RT-PCR. A total of 117 samples were collected for virus detection by a one-step RT-PCR protocol (Kundu, 2003), and multiplex RT-PCR (Menzel *et al*., 2002, 2003). Briefly, plant materials (leaves or bark) were ground in liquid nitrogen and total RNA was extracted with a RNAeasy plant kit (Qiagen, Germany) according to manufacturer's instructions. Multiplex RT-PCR was performed with a one-step RT-PCR kit (Qiagen, Germany) using specific detection primers (Menzel *et al.*, 2002). The PCR products were analyzed in 1.5% agarose gel electrophoresis and visualized under UV light.

A total of 35 isolates, 13 ACLSV, 13 ASPV and 9 ASGV were selected for sequencing and genetic analysis (Table 2). cDNA was obtained by RT using following virus- specific reverse primers: (i) ACLSV, 5'-AAGTCTACAGGCTATTTATTATAAGTCTAA-3'; (ii) ASPV, 5'-GTCCCGGTTAGGTTGGGATC-3'; (iii) ASGV, 5'-GAGTGGACAAACTCTAGACTC-3'. A mix of 1x buffer, 4 U of *Avian myeloblastosis virus* (AMV) reverse transcriptase, 1 µM of each dNTPs (Promega, USA), 0.25 µM of each primer and 2 µl of RNA was incubated at 42°C for 1 h, kept for 2 min at 95°C for enzyme inactivation, then chilled on ice.

Two sets of degenerate primer pairs were newly designed, based on sequences retrieved from GenBank, to amplify the selected region of ASPV and ASGV (Table 3), whereas, for ACLSV, a reverse primer designed by Menzel *et al.* (2002) was used together with a newly designed forward primer ACLSVFrII. The position of target sequences of all three sets of primer pairs used is shown in Table 3.

The PCR reactions were performed with ExTaq^{TM} (TaKaRa, Japan) carried out in final volume of 50 µl, containing 1x buffer ExTaq^{TM}, 250 µM of each dNTPs, 1 µM of each primer, 1 U of ExTaq^{TM} (TaKaRa) and 3 µl of cDNA. The PCR conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for elongation. Final elongation was at 72°C for 5 min. PCR fragments of 798, 840 and 820 bp were obtained for ACLSV, ASPV and ASGV, respectively.

DNA sequencing and genetic analysis. ACLSV-derived and ASGV-derived PCR products were sequenced (Macrogen, Korea) and the final contigs were edited with Sequencher 4.8 (Gene Codes Inc, USA). PCR products of 13 ASPV isolates and three isolates of ACLSV and ASGV were cloned into pGEM-T easy vector and sequenced with M13 universal primers. Our virus-specific sequences and those from GenBank (including members of genera *Trichovirus*, *Capillovirus* and *Foveavirus*) were aligned using Clustal-X (Thompson *et al*., 1997). For phylogenetic studies, three matrices of 572, 729 and 802 bp were chosen for *Trichovirus*, *Capillovirus* and *Foveavirus*, respectively. Three different analyses were performed for each genus. The Neighbor-joining method (Saitou and Nei, 1987) was used with Clustal W.

RESULTS AND DISCUSSION

Virus detection. ACLSV, ASGV and ASPV were detected in 108 of 117 tested pome fruit samples from seven countries (Table 1) using primers described by Menzel *et al.* (2002). The high virus incidence was in agreement with a previous study carried out in commercial orchards and nurseries in the Czech Republic (Kundu, 2003a). Similarly, the high occurrence of mixed infections confirmed previous reports (Leone *et al*., 1998; Kundu, 2003b).

Sequence and phylogenetic analysis. Sequencing of the ACLSV-, ASPV- and ASGV-specific PCR products from the 35 isolates under study confirmed their viral origin. Comparison of sequences of the C-terminus of the MP and the N-terminus of the CP genes (6,921-7,407 nt) of isolates for which sequences are available in GenBank showed that all ACLSV isolates represent a monophyletic group, clearly supported by the bootstrap values, separated from other members of the genus *Tri-*

Table 2. Origin, host and typing of the isolates characterized in this study.

Name	Origin	Host	Typing	Accession Nos
5SrACL	Serbia	Apple cv. Kolacara	ACLSV	FJ952172
8SrACL	Serbia	Apple cv. Budimka	ACLSV	FJ952173
9SrACL	Serbia	Apple cv. Stark's Earliest	ACLSV	FJ952178
11SrACL	Serbia	Apple cv. Granny Smith	ACLSV	FJ952177
14SrACL	Serbia	Apple cv. Delbar	ACLSV	FJ952174
E13ACL	Czech Republic	Apple cv Early Smith	ACLSV	FJ952168
E15ACL	Czech Republic	Apple cv Early Smith	ACLSV	FJ952169
J11ACL	Czech Republic	Apple cv. Jonagold	ACLSV	FJ970962
JI-IACL	Poland	Apple	ACLSV	FJ952175
5BeACL	Belgium	Apple	ACLSV	FJ952176
7UkACL	Ukraine	Apple	ACLSV	FJ952170
13UkACL	Ukraine	Apple cv. Champion	ACLSV	FJ952171
TK4ACL	Turkey	Apple	ACLSV	FJ952167
E13ASP	Czech Republic	Apple cv Early Smith	ASPV	FJ970958
E15ASP	Czech Republic	Apple cv Early Smith	ASPV	FJ970959
A3ASP	India	Apple	ASPV	FJ970956
JI-IASP	Poland	Apple	ASPV	FJ970957
J30-IASP	Poland	Apple	ASPV	FJ970949
398ASP	Belgium	Apple	ASPV	FJ970953
402ASP	Belgium	Apple	ASPV	FJ970954
405ASP	Belgium	Apple	ASPV	FJ970955
7UkASP	Ukraine	Apple	ASPV	FJ970950
8UkASP	Ukraine	Apple	ASPV	FJ970951
11UkASP	Ukraine	Apple cv. Jonagold	ASPV	FJ970952
TK3ASP	Turkey	Apple	ASPV	FJ970960
TK4ASP	Turkey	Apple	ASPV	FJ970961
19SrASG	Serbia	Apple cv. Granny Smith	ASGV	FJ952163
TK4ASG	Turkey	Apple	ASGV	FJ952166
TK7ASG	Turkey	Apple	ASGV	FJ952158
A5ASG	India	Apple	ASGV	FJ952159
A6ASG	India	Apple	ASGV	FJ952160
A7ASG	India	Apple	ASGV	FJ952164
A8ASG	India	Apple	ASGV	FJ952165
E13ASG	Czech Republic	Apple cv Early Smith	ASGV	FJ952161
E30ASG	Czech Republic	Apple cv Early Smith	ASGV	FJ952162

Table 3. Primers used in this study.

Primers	Position	Sequences	References
RT-primer ACLSV	7507-7536	AAGTCTACAGGCTATTTATTATAAGTCTAA	Menzel *et al.*, 2002
ACLSVFrII	6745-6766	CAAGAGAATTTCAGTTTGCTCG	This study
ASPV forward	8312-8330	CWAAYCCWTTTGAAACTGG	This study
ASPV reverse	9134-9151	GCTTGGGTCCAAYYTTTC	This study
ASGV forward	5644-5661	GTTTGGAAGACGTGCTTC	This study
ASGV reverse	6446-6463	ACACTAACCCGGAAATGC	This study

chovirus (Fig. 1). A high molecular divergence among ACLSV isolates was found, at the nucleotide (nt) level, identity ranging from 83.5 to 85.0%, (Fig. 1), a value in line with that estimated for other trichoviruses (73.9-84.2% nt for all trichoviruses in this study). The closest phylogenetic relationship was between two Ukrainian ACLSV isolates (7UkACL and 13UkACL). However, five isolates from Serbia clearly grouped apart from each other (Fig. 1). These results tally with previous observation (Al Rwahnih *et al.*, 2004) reporting that the end of the 50-kDa protein coded by ORF-2 (MP) and the beginning of the CP gene had the highest variability.

The ACLSV group B found by Al Rwahnih *et al.* (2004) was also present in our analyses (Cluster 1 in Fig. 1).

The ASGV-specific amplified fragment (630 bp) corresponding to the CP gene from nine isolates was sequenced and analyzed. Nucleotide sequence identity among ASGV isolates (Fig. 2) ranged from 90.6 to 91.8%, very similar to those within and among the other members of the genus *Capillovirus* (80.9-82.1%). The

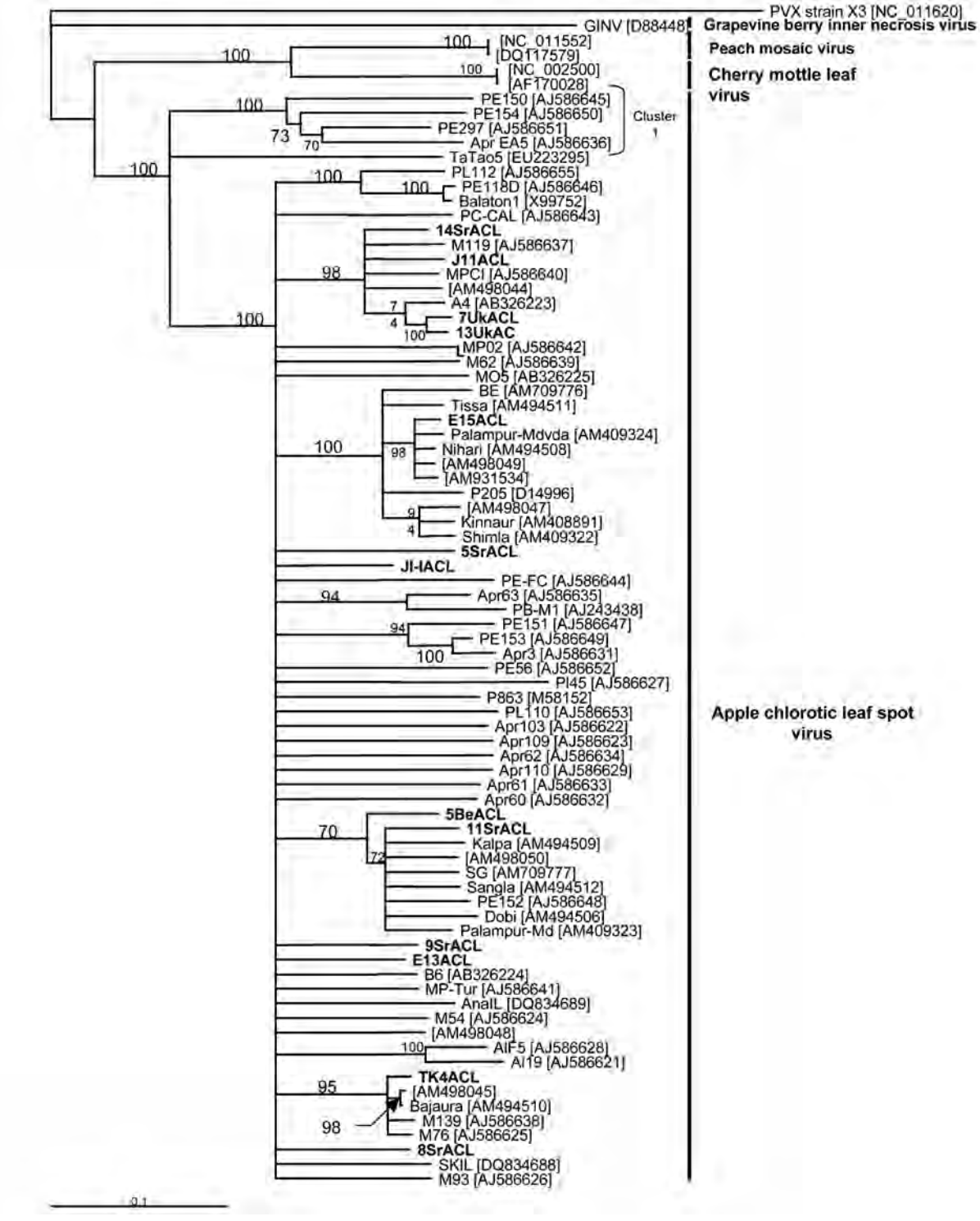

Fig. 1. Phylogenetic tree constructed with sequences comprising the C-terminus of the movement protein gene and the N-terminus of the coat protein gene of isolates of *Apple chlorotic leaf spot virus* (ACLSV), *Peach mosaic virus* (PcMV), *Grapevine berry inner necrosis virus* (GINV) and *Cherry mottle leaf virus* (CMLV), genus *Trichovirus*. Nodes bearing <70% bootstrap values support were collapsed to polytomies. Only bootstrap values higher than 70% are shown. Branches length corresponding to the genetic distances as indicated on the scale bar. The strain X3 of *Potato virus X,* genus *Potexvirus* (NC_011620) was used to root the tree. The name of the isolates and their accession numbers are indicated in the tree. The samples analyzed here are highlighted in bold.

phylogenetic tree (Fig. 2) showed that the ASGV isolates were clearly separated from *Cherry virus A*, another member of the same genus. ASGV isolates separated into two groups, the first of which (Cluster 1 in Fig. 2) comprises the majority of samples, and were closely related to an Indian (Accession No. FM204881) and a Brazilian isolate (UV01, accession No. AF438409). Several isolates of *Citrus tatter leaf virus* (Yoshikawa *et al.*,

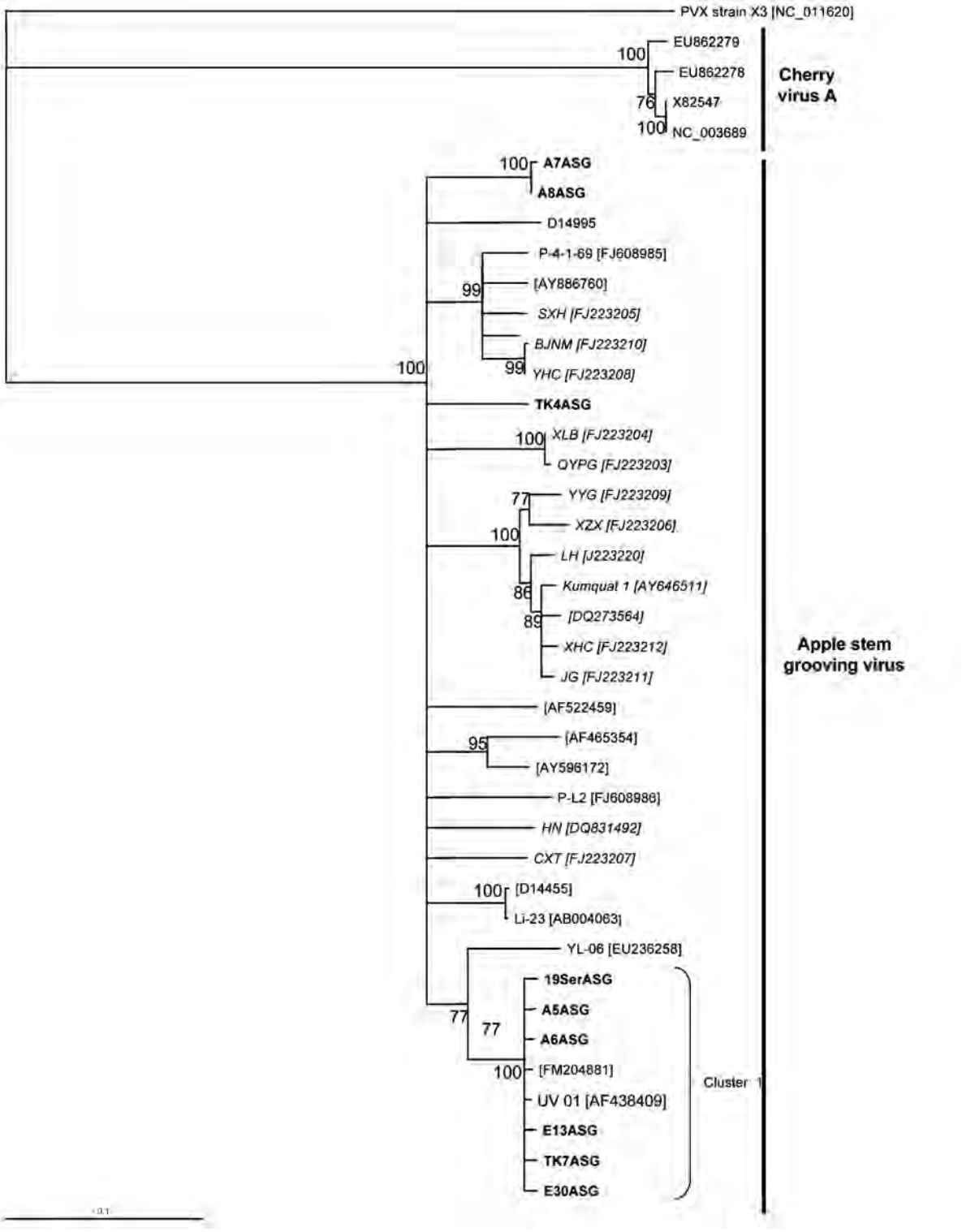

Fig. 2. Phylogenetic tree contructed with the coat protein gene sequences of isolates of *Apple stem grooving virus* (ASGV) and *Cherry virus A* (CVA), genus *Capillovirus*. Nodes bearing <70% bootstrap values support were collapsed to polytomies. Only bootstrap values higher than 70% are shown. Branches' length corresponding to the genetic distances as indicated on the scale bar. The strain X3 of *Potato virus X,* genus *Potexvirus* (NC_011620) was used to root the tree. The name of the isolates and their accession numbers are indicated in the tree. The samples analyzed here are highlighted in bold and CTLV isolates are in Italics.

1993) were included in our analysis (italics script in Fig. 2) but could not be clearly discriminates from those of ASGV, consistently with results of Ohira *et al.* (1995) and Nickel *et al.* (2001).

The genetic diversity of 13 ASPV isolates was studied by sequencing of the CP region (8,322-9,133 bp) and aligning a matrix of 802 bp with data of other ASPV isolates from GenBank. The analysis also included several members of *Foveavirus*: Peach sooty ring spot virus (AF318062), *Apple latent virus* (AF057035) and Peach

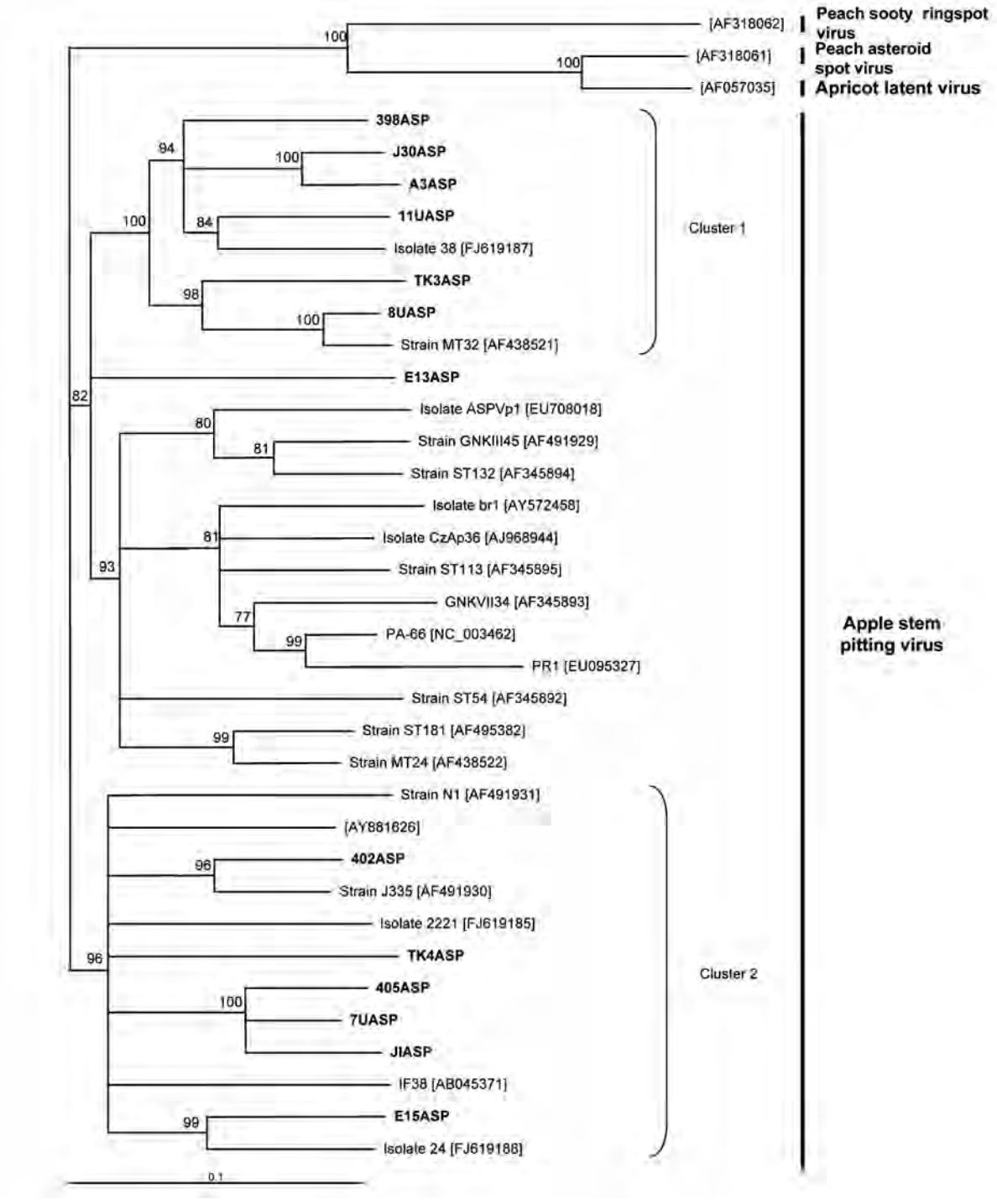

Fig. 3. Phylogenetic tree constructed with sequences of the coat protein gene of isolates of *Apple stem pitting virus* (ASPV), Peach sooty ringspot virus (PSRV)*, Apricot latent virus* (ApLV)*,* Peach asteroid spot virus (PEASV), genus *Foveavirus.* Nodes bearing <70% bootstrap values support were collapsed to polytomies. Only bootstrap values higher than 70% are shown. Branches length corresponding to the genetic distances as indicated on the scale bar. Peach sooty ringspot virus (AF318062), *Apricot latent virus* (AF057035) and Peach asteroid spot virus (AF318061) were used to root the tree. The name of the isolates and their accession numbers (in bracket) are indicated in the tree. The samples analyzed here are highlighted in bold.

asteroid spot virus (AF318061) which were used to root the tree. Among ASPV isolates sequence identity at the nucleotide level ranged from 80.1 to 81.9% (Fig. 3). The ASPV isolates grouped into two major clusters, the first comprising eight isolates, six of which were studied in this work (398ASP, J30ASP, A3ASP, 11UASP, TK3ASP and 8UASP; Cluster 1 in Fig. 3). The second cluster (Cluster 2 in Fig. 3) comprised six isolates (402ASP, TK4ASP, 405ASP, 7UASP, JIASP and E15ASP). One isolate (E13ASP) clustered apart from both clusters.

There was no correlation between the geographic origin of the ACLSV, ASGV and ASPV isolates and their genetic diversity, which does not allow to draw conclusions on their origin and dispersion. However, the high incidence of infections emphasizes the need for certification schemes for the production of virus-free propagating material.

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