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Molecular typing of Turkish *Apple chlorotic leaf spot virus* isolates based on partial coat protein gene

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Summary. *Apple chlorotic leaf spot virus* (ACLSV) isolates from various hosts and geographic locations in Turkey were molecularly characterized by RFLP, nucleotide sequence analysis and the construction of a phylogenetic tree including ACLSV isolates from GenBank. Based on nucleotide sequence alignment and the phylogenetic tree, we proposed a classification of ACLSV isolates in which isolates were divided into three major groups. The first group contained mainly Far-Eastern isolates, the second group the Hungarian (eastern-European) ACLSV isolates, and the third group, which contained isolates of variable characteristics, was again divided into two subgroups, subgroup I containing mixed European isolates, and subgroup II containing central European isolates. Three representative Turkish ACLSV isolates belonged to the third group; of these, one was from the mixed European cluster (subgroup I) and two from the central European cluster (subgroup II). The nucleotide sequence divergence and geographic origin of the ACLSV isolates were correlated, which indicated the possible extraction of the Turkish isolates.

Key words: ACLSV, RT-PCR, stone fruits.

Introduction

Apple chlorotic leaf spot virus (ACLSV) occurs world-wide on various fruit tree hosts including apple, peach, pear, plum, cherry and apricot, and also on many ornamental species (Nemeth, 1986; Desvignes and Boye, 1988). Although it is mostly symptomless in many of its host plants, ACLSV may also cause serious diseases, producing graft incompatibility in certain scion and rootstock combinations. When ACLSV causes symptoms, they include apple russet ring, dark-green sunken mottle, and severe leaf deformation on peach leaves (butteratura) and on apricot fruits (viruela), local necrosis on plums (false plum pox on fruits) and bark split (Desvignes and Boye, 1988). Damage to crops and weakening of trees are also caused by certain virus isolates, resulting in a gradual decline. Although the economic significance of the virus is very variable, the spread of ACLSV is limited by the apparent absence or ineffectiveness of natural vectors for this virus.

Complete nucleotide sequences of several ACLSV isolates have been determined (German *et al.*, 1990; Sato *et al.*, 1993; Jelkmann, 1996; German-Retana *et al.*, 1997; Malinowski *et al.*, 1998). A knowledge of these sequences has facilitated the detailed analysis, comparison, and specific detection of the virus. The results of these studies suggest that ACLSV is a type member of *Trichovirus* (Martelli *et al.*, 1994), its genome

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consisting of a single strand polyadenylated RNA molecule of 7545–7555 nucleotides, excluding the poly-A tail, with three open reading frames (ORF 1, 2 and 3) encoding three proteins of 216.5 kDa, 50 kDa and 28.3 kDa respectively, and lacking intergenic regions (German *et al*., 1990). The 216.5 kDa protein contains two consensus sequences associated with RNA polymerase and NTP-binding helicase. The 50 kDa protein is suggested to be the movement protein, and the 28.3 kDa product the viral coat protein (German *et al*., 1992).

Although the biological variability of ACLSV isolates is well documented (Desvignes and Boye, 1988), information on molecular differences between virus isolates is limited. Candresse *et al*. (1995) described a group of several ACLSV isolates originating in Europe (France, Hungary, Poland, and Germany) and one in Japan, and found that these isolates were grouped into two main clusters consisting of the French prune isolate and the central European isolates originating in various host plants. Pasquini *et al*. (1998) classified the Italian ACLSV isolates on the basis of the electrophoretic mobility of the coat proteins (CP) into three main groups, with an apparent Mr of 22.7 kDa, 21.5 kDa and 19.7 kDa respectively. The Hungarian ACLSV isolates were also classified into three groups by the electrophoretic mobility of their CP (Krizbai *et al.,* 2001). Al Rwahnih *et al*. (2004) investigated the phylogenetic relationships of a larger number of isolates from various host species and geographic origins. Like Candresse *et al*. (1995), they also classified isolates into two well-defined groups: the first group included the Italian, Hungarian, Turkish and Jordanian isolates, and the second group the remaining isolates.

The occurrence of ACLSV on stone fruit trees in Turkey has been known since the 1990s (Azeri, 1994). Turkish isolates, however, have not been molecularly characterized. In the present study, we determined a partial sequence of the coat protein gene of three representative ACLSV isolates from Turkey and applied molecular typing methods to characterize them. These isolates were compared with other ACLSV isolates from the GenBank database and were classified into groups in order to gain information on the molecular variability, interrelationship and possible origin of these isolates.

Materials and methods

Virus isolates

The three virus isolates, their host plants, the location in Turkey where they were collected and the GeneBank accession numbers of the three ACLSV sequences described below are listed in Table 1. These isolates were selected from various samples obtained from several host plants growing in different regions of Turkey. The other ACLSV isolates which were studied are listed in Table 2.

RNA extraction

Four to six shoots were picked from each tree in the second half of May and preserved dry in $CaCl_2$ as frozen tissue or their nucleic acids were immediately extracted. A 100-mg sample of fresh material, or 25 mg of dried tissue, was homogenized in 5 volumes of LiCl buffer and the nucleic acids were extracted using 6 M potassium acetate (pH 6.5) and isopropanol precipitation (Spiegel *et al*., 1996). The dried pellet was washed with ethanol and suspended in 50 μl sterile water. One μl of RNA was used as a template for reverse transcription-polymerase chain reaction (RT-PCR).

Primers and RT-PCR

The universal primers A52 and A53 were used in RT-PCR of the ACLSV sequences (Candresse *et al*., 1995). The reverse transcription primer (A52) had a sequence of 5'-CAGACCCTTATTGAAGTC-

Table 1. Description of Turkish *Apple chlorotic leaf spot virus* isolates.

Accession number	Isolate	Host	Variety	Location in Turkey
AY730558	KP2	Peach	Dixired	Bursa
AY730559	AP10	Peach	Unknown	Amasya
AY730560	ASwC43	Sweet cherry	Unknown	Amasya

Table 2. *Apple chlorotic leaf spot virus* isolates referred to and described in other sources.

Accession number	Isolate	Host	Location	Reference
AF251275	SX/2	Plum	Poland	Malinowski *et al*., 1996
AJ243438	PBMI	Plum	Germany	Jelkmann *et al*., 1996
AY669389	Kuerle	Pear	China	Cai *et al*., unpublished
AY677103	AT43	Apple	Hungary	Krizbai *et al*., unpublished
AY677104	AT49	Apple	Hungary	Krizbai *et al*., unpublished
AY677105	C1	Wild cherry	Hungary	Krizbai *et al*., unpublished
AY677106	C2	Wild cherry	Hungary	Krizbai *et al*., unpublished
AY677107	P1	Peach	Hungary	Krizbai *et al*., unpublished
D14996	P205	Apple	Japan	Sato *et al*., 1993
M58152	P863	Plum	France	Germann *et al*., 1990
X99752	Bal1	Wild cherry	Hungary	German-Retana *et al*., 1997

GAA-3' (derived from positions 7213–7233 nts of the ACLSV sequence, GenBank accession No. M58152). The sense primer (A53) sequence was 5'-GGCAACCCTGGAACAGA-3' (position 6875–6891 nts). The amplified product was 358 bp in length.

RT-PCR amplification was carried out in 50 μl of reaction mixture containing RNA template (about 1 μg), virus-specific primers (0.8 μM each), 400 μM dNTPs, 1.5 mM $MgCl_2$, 10× reaction buffer (final concentration of 10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.08% Igepal), 1 unit of Taq DNA polymerase (Promega, Madison, WI, USA), 0.8 unit of M-MLV reverse transcriptase (Fermentas GmbH, Germany) and 0.8 unit of RNase inhibitor (Fermentas). The thermal cycling protocol of the reaction was as follows: one hour incubation at 42°C for reverse transcription, 35 cycles of 30 s at 94°C, 45 s at 55°C, 1 min at 72°C, and finally 10 min at 72°C. The PCR products were fractionated by electrophoresis in a 1% agarose gel, stained with ethidium bromide (EtBr) and visualised under UV.

Restriction endonuclease analysis

Ten μl samples of the amplified PCR products were digested with 5 U of *Eco*RI, *Hinc*II, *Bam*HI, *Taq*I, *Apa*I and *Rsa*I (Fermentas) in a final volume of 15 μl containing buffer and water according to manufacturer's instructions. The mixtures were incubated overnight at 37°C, except for *Taq*I which was incubated at 65°C. The digests were fractionated by electrophoresis in 6% polyacrylamide gel using TAE buffer. The gels were stained with EtBr.

Cloning and sequencing of RT-PCR products

Following amplification, the PCR products were purified by agarose gel electrophoresis in 1× TAE buffer and purified using a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The purified products were then cloned into a pGEM-TEasy plasmid vector (Promega). The ligation mixture was used to transform *Escherichia coli* cells (strain JM109). Bacterial cells carrying recombinant plasmids were selected and the plasmid clones were sequenced from both ends using the T7 and SP6 primers at the Nucleotide Sequencing Centre (Rehovot, Israel).

The nucleotide sequences of the Turkish isolates were submitted to the GenBank and registered under accession numbers AY730558, AY730559 and AY730560.

Phylogenetic analysis

Multiple alignments of nucleotide and amino acid sequences of the ACLSV CP gene were carried out using the default options of Clustal X 1.8, a Windows interface for the Clustal W multiple sequence alignment program (Higgins *et al*., 1994). The alignments were used as input data to construct phylogenetic trees using the neighbor-joining method on a Macintosh with the computer program PAUP (phylogenetic analysis using parsimony), 4.0 Beta Version (Swofford, 1993). Bootstrap analysis with 1000 replicates was performed to estimate the support for the inferred phylogenies.

Results and discussion

RFLP analysis of ACLSV isolates

To molecularly characterize the Turkish ACLSV isolates, eight isolates (KP2, KP4, KP5, AP10, AP11, AP62 from peach, and ASwC43 and ASwC44 from sweet cherry) were selected and characterized using RT-PCR-RFLP analysis. The PCR products (about 358 bp) of the various isolates were digested by four restriction endonucleases (Fig. 1). Two main patterns of cleavage of the ACLSV isolates were observed. The first included isolates KP2, KP4, and KP5, cleaved only by *Hinc*II. The second (isolates Ap10, AP11, ASwC43 and ASwC44) were cleaved by *Hinc*II and by *Bam*HI and *Taq*I. The ASwC43 isolate which belonged to the second group was also cleaved by *Eco*RI, while isolate AP62 was cleaved by both *Hinc*II and *Bam*HI, but not by *Taq*I or *Eco*RI. Three Turkish isolates: KP2, AP10 and ASwC43, which produced different restriction patterns, were chosen for further molecular characterization.

Sequence analysis

The nucleotide sequences of the three Turkish ACLSV isolates (Fig. 2) covered nucleotides 94–452 (M58152) of the 3'-end half of the 50 kDa and 28 kDa ORFs overlapping regions of the ACLSV-CP. No nucleotide insertion or deletion was found among the ACLSV isolates in multiple nucleotide sequence alignment. Similarly, multiple nucleotide sequence alignments of immuno capture-RT-PCR amplified products with A52/A53 primers of ACLSV-Cen, Cis and Bit isolates exhibited no nucleotide insertion or deletion (Pasquini *et al*., 1998). To our knowledge, this is the first description of the nucleotide sequence of an ACLSV isolate from sweet cherry (ASwC43).

When the nucleotide sequence of the CP (partial) gene of ACLSV-Kuerle (from China) was compared with the Turkish isolates KP2, AP10 and ASwC43, the identity of the latter with the CP gene was low: 79.61, 79.89 and 79.79% respectively (Table 3). A greater degree of identity (93.02%) was found between the peach and plum isolates, KP2 from Turkey and P863 from France. The nucleotide acid sequence homology for the Turkish ACLSV isolates confirmed Candresse *et al*. (1995) who reported a high sequence divergence (77.4 and 99.4 %.) between ACLSV isolates.

German-Retana *et al*. (1997) found that most of the variations between ACLSV isolates were located within three regions: a hyper-variable segment downstream of the methyl-transferase domain of 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 CP coding region. Alignment of the putative amino acid sequences (Fig. 3) showed that variability was higher at the N-terminal than at the C-terminal part of the CP gene as was previously reported (Candresse *et al*., 1998; Al Rwahnih *et al*., 2004). The Turkish isolates AP10 and ASwC43 differed from all other isolates in having two unique amino acids: t-threonine and l-leucine, at positions 49 and 73 respec-

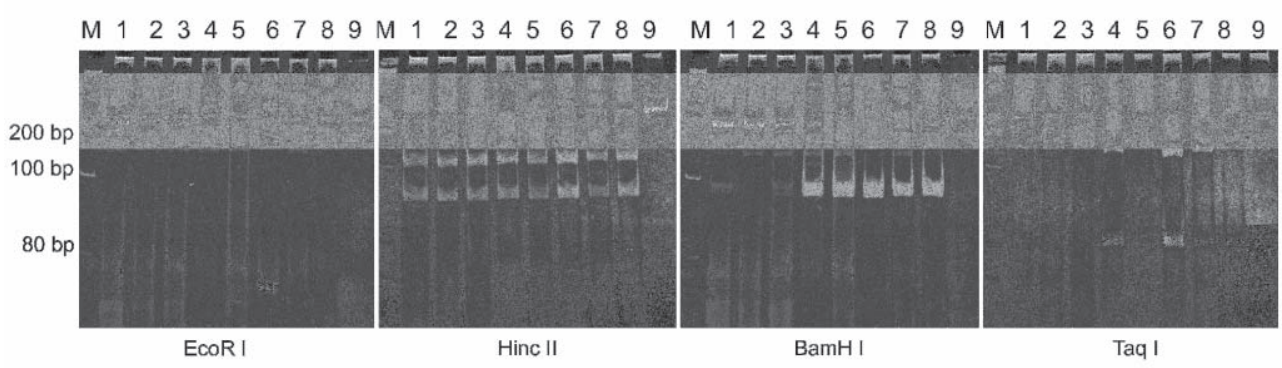

Fig. 1. Restriction fragment length polymorphism analysis of PCR products of eight Turkish *Apple chlorotic leaf spot* isolates. M, molecular weight marker (Lambda DNA/ *Eco*RI +*Hind*III, Marker 3, Fermentas GmbH, St. Leon-Rot, Germany); 1, KP2; 2, KP4; 3, KP5; 4, AP10; 5, AP62; 6, ASwC43; 7, ASwC44; 8, AP11; 9, control-DNA with no added enzyme.

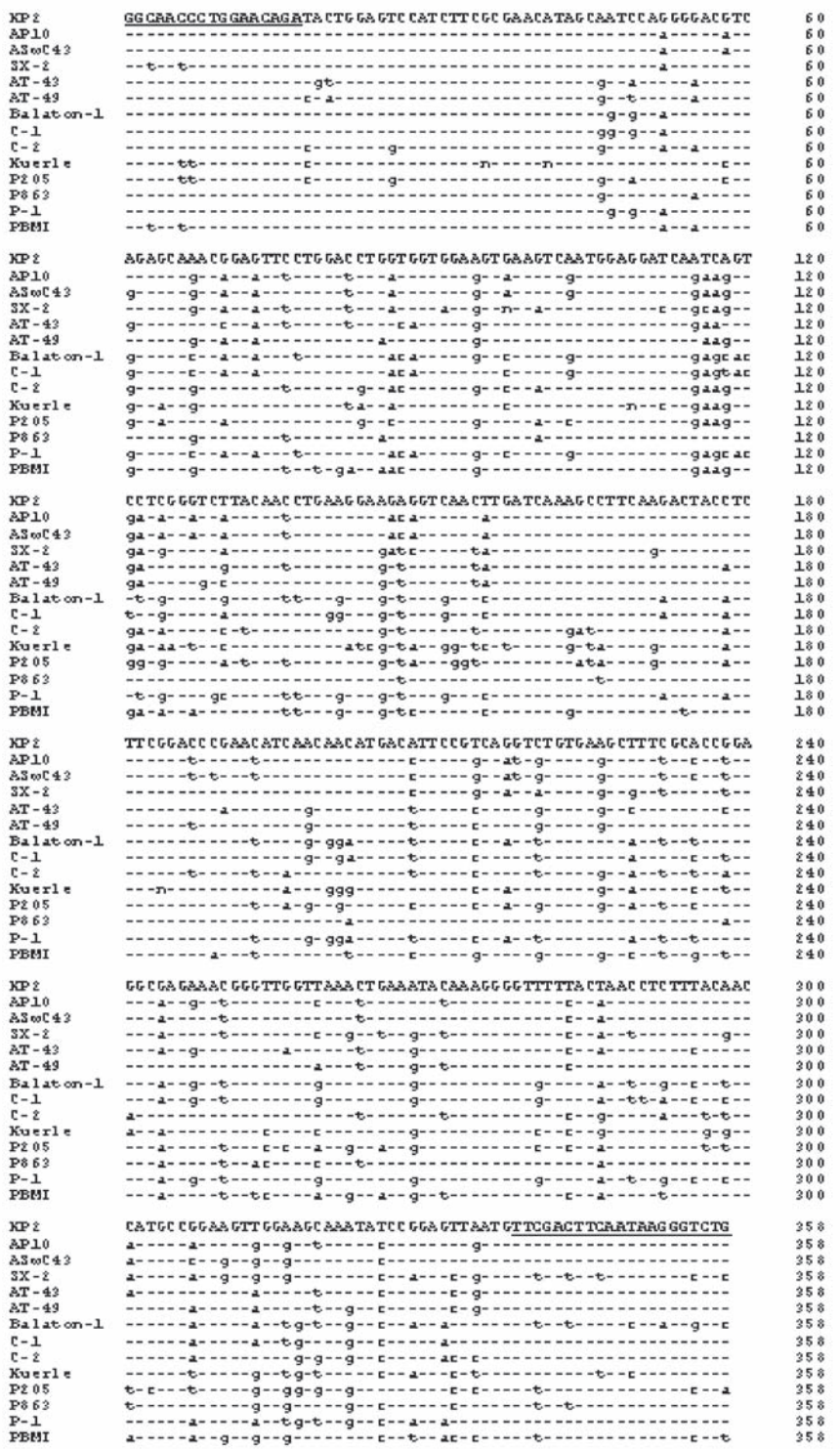

Fig. 2. Multiple nucleotide sequence alignment of *Apple chlorotic leaf spot virus* isolates based on the 3'-end region of the 50 kDa and 28 kDa proteins of the overlapping ORF. Dashes indicate conserved nucleotides. The upper underlined sequence represents the A53 primer, the lower one, the A52 primer.

tively. The amino acid sequence of isolate KP2 was very similar to that of isolate P863, which originated in France. The homology of the amino acid sequences between the ACLSV isolates (Table 3) varied from 84.03% (isolates P-205 vs. ASwC43) to 98.32% (P863 vs. KP2). The putative polypeptide sequences of isolates P-205 (from Japan) and Kuerle (from China) exhibited the highest degree of sequence diversity.

These results may explain the high level of bio-

Table 3. Percentage of identity of nucleic acid (na) and amino acid (aa) sequences between pairs of *Apple chlorotic leaf spot virus* isolates. Identities of interest are given in bold numbers.

GenBank Isolate	Turkish isolate					
	KP2		AP10		ASwC43	
	na (%)	aa (%)	na (%)	aa (%)	na (%)	aa (%)
SX/2	82.68	93.28	88.83	94.12	87.71	93.28
PMB1	81.28	91.60	86.03	91.60	86.31	91.60
Kuerle	**79.61**	84.87	**79.89**	85.71	**79.89**	84.82
AT-43	86.87	94.12	86.59	94.96	85.75	94.12
AT-49	89.11	93.28	87.43	93.28	86.03	92.44
C-1	83.80	92.40	82.12	88.24	82.40	87.39
C-2	84.08	90.76	84.36	89.92	83.80	89.08
P-1	81.84	91.60	82.40	87.39	82.12	86.55
P-205	79.33	85.71	81.01	84.87	81.56	**84.03**
P863	**93.02**	**98.32**	84.92	93.28	84.64	92.44
Balaton-1	80.45	92.44	81.01	88.24	80.73	87.39

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KP2         ATLEQILESIFANIAIQGTSEQTEFLDLVVEVKSMEDQSVLGSYNLKEEVNLIKAFKTTS     60
AP10        ----------------------------m---------k-i-------t--m--------     60
ASwC43      ----------------------------m---------k-i-------t--m--------     60
Balaton-1   ---------------v-----h------t----------t------r-v------------     60
P863        ------------------------------------------------v------------     60
PBMI        vi----------------------g-t---------k-i-----r-v--------i--     60
AT-43       -----m----------------------a---------k-i-------v--m--------     60
AT-49       -----tm---------------------------------k-i-a-----v--m--------     60
C-1         ---------------v-----h------t----------t------g-v------------     60
C-2         -----t--a-------------------vt---------k-i--f----v-----i------     60
P205        -i---t--a-------------------vl---------k-v--f----v-g---i-r---     60
P-1         ---------------v-----h------t----------t--c---r-v------------     60
SX-2        vi----------------------------m---------q-m-------i--m----r---     60
Kuerle      -i---t-----x-x-------------mm------x--k-is-----sv-d---v-r---     60

KP2         SDPNINNMTFRQVCEAFAPEARNGLVKLKYKGVFTNLFTTMPEVGSKYPELMFDFNKGL     119
AP10        ------------l-------------------------------------------     119
ASwC43      --l---------l-------------------------------------------     119
Balaton-1   -----sg-------------------------------------g------------     119
P863        ------k-------------------------------------------------     119
PBMI        --q-----------------------------------------------------     119
AT-43       -----s---------------------------------------------------     119
AT-49       -----s---------------------------------------------------     119
C-1         -----sr-------------------------------------g------------     119
C-2         ------------------------------------s------g------------     119
P205        -----ss-----------------------------s-t----g------------     119
P-1         -----sg-------------------------------------g------------     119
SX-2        -------------------------------------------------------     119
Kuerle      ------g----------------------------a------g------------     119
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Fig. 3. Multiple amino acid sequence alignment of *Apple chlorotic leaf spot virus* isolates in the region of the coat protein gene. Dashes indicate conserved amino acids.

logical variability found between ACLSV isolates. As suggested by Candresse *et al*. (1995), the high degree of biological variability could be the result of sequence divergence at the 54K movement protein, while the serological similarity between ACLSV isolates could be explained by the low variability in the coat protein gene which appeared to be the most conserved of all ACLSV polypeptides (German-Retana *et al*., 1997).

A phylogenetic tree was generated on the basis of the nucleotide sequence data obtained (Fig. 4). ACLSV isolates were classified into three main groups based on their nucleotide sequence identity. This grouping was well supported by bootstrap analysis. The first group (I) contained isolates P-205 (Japan), Kuerle (China) and C2 (Hungary). It should be noted that besides the C2 isolate from Hungary, all the isolates in this group originated in the Far-East, and that each of these isolates had a different host (apple, pear and wild cherry, respectively). Similarly, Candresse *et al*. (1995) classified an apple isolate from Japan as belonging to a separate group of uncharacterized isolates. It is surprising however, that the Hungarian wild cherry isolate was grouped with these Far-Eastern isolates, and this raises questions about the geograph-

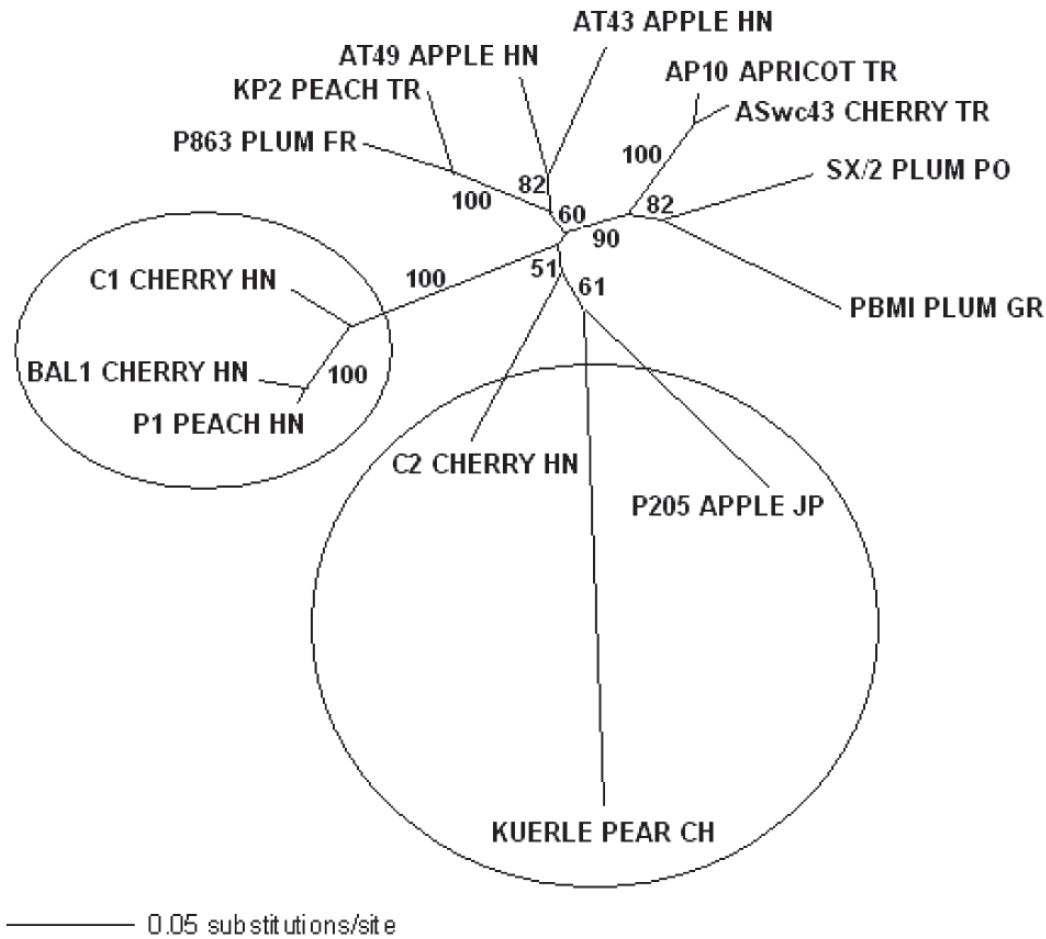

Fig. 4. Unrooted phylogram showing the relationships of the amplified product of Turkish *Apple chlorotic leaf spot virus* isolates and sequences from GenBank at the nucleotide level. The phylogram was constructed using the neighbor-joining method on the Power Macintosh computer program PAUP. The numbers near the nodes were determined by bootstrap analysis with 1000 replicates in percentage, and are only shown when they exceed 50%. PO, Poland; GR, Germany; CH, China; HN, Hungary; JP, Japan; FR, France; TR, Turkey.

ic origin of the Hungarian isolate. The second group (II) included three Hungarian isolates: C1, Bal1 and P1 (the first two from wild cherry, the last from peach). These stone-fruit isolates were distinct from the pome-fruit isolates (AT-42 and AT-43). The third group (III) included highly variable isolates and consisted of Turkish (KP2, AP10 and ASwC3), German (PBMI), Polish (SX/2), French (P863) and Hungarian (AT-42 and AT-43) isolates from peach, sweet cherry, plum and apple. This group could be further divided into two subgroups: the first subgroup consisting of a mixed European cluster with two Hungarian apple isolates (AT43 and AT49) a Turkish peach isolate (KP2) and a French plum isolate (P863), and the second subgroup being a central European cluster that contained two isolates from Turkey (ASwC43 from sweet cherry, AP10 from peach) and plum isolates from Germany (PMBI) and Poland (SX/2).

In a recent study the nucleotide sequence of 35 ACLSV isolates, including two Turkish isolates, from different pome and stone fruit hosts and geographic locations, was determined and a phylogenetic analysis at the nucleotide and amino acid levels clustered these isolates into two groups (Al Rwahnih *et al*., 2004). In that study, an apple isolate from Turkey was classified in Group A, together with Italian, Hungarian and Jordanian peach ACLSV isolates. An apricot isolate from Turkey was clustered in Group B, which also contained the great majority of isolates from different hosts and originating countries. On the basis of the nucleotide sequence alignment and a phylogenetic tree, we obtained a slightly modified mode of classification of ACLSV isolates, in which isolates were divided into two main groups; one containing mainly Far-Eastern isolates and one that included the Hungarian (east-European) ACLSV isolates. A third group containing isolates of variable characteristics was divided into two subgroups, I, including mixed European isolates, and II, including central European isolates (see Fig. 4). We showed that the two Turkish ACLSV isolates (AP10 and ASwC43) belonged to the central European cluster, which differed markedly from the second subgroup of the mixed European cluster, in which the Turkish peach isolate (KP2) was classified. This may give an indication of the origin of these isolates. There was a correlation between nucleotide sequence divergence and the geographic origin of the ACLSV isolates, and this may give an indication of extraction of the Turkish isolates.

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