Indian Journal of Biochemistry & Biophysics
Vol. 56, December 2019, pp. 514-519

Genome cloning and genetic diversity of *Apple chlorotic leaf spot virus*

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Received 24 January 2019; *revised 17 April 2019*

Apple chlorotic leaf spot virus (ACLSV) with a wide distribution and variability is great threat to apple yield and quality. The systematic research on the occurrence, genetic structure and evolutionary mechanism is important for the prevention of ACLSV. In this study, 360 apple leaf samples were collected from Shanxi province and tested by RT-PCR, and the result showed that the incidence of ACLSV in Shanxi was ranged from 43.59% in Linfen to 68.18% in Wanrong. One new ACLSV isolate (shanxi14-MK368727) was collected from the positive samples, of which the genome (including the 5' and 3' ends) was 7507 bp and encoded 2536 amino acids. Compared with online database, the highest nd identity was between shanxi14 and KJ522693.1, and the lowest was shanxi14 and M58152.1. Phylogenetic analyzed based on genome showed that 25 isolated of ACLSV were divided two groups (Group I and II), which showed that was no significant correlation with geographic location. The selection pressures of POL, MP and CP were tested, the result proved the three genes were under negative selection pressure. The knowledge presented in this study will be useful in for the design of long-term, sustainable management strategies for controlling these viruses.

Keywords: *Apple chlorotic leaf spot virus* (ACLSV), Genetic diversity, Genome clone, Selection pressure

Apples is one of four major fruits in the world[1,2], and distributed widely in many countries such as China, Europe, Japan, Australia and American[3]. According to the statistics of Food and agriculture organization of the united nations, the total planting area was about 4.4 million hectares (ha) and output was about 65 million tons (tonne) of the world in 2011. The occurrence and development of apple virus disease seriously affects the quality and yield of apple, causing huge economic losses in apple commercialize production areas[4,5]. According to the statistics of the China National Bureau of Statistics in 2014, virus is one of the most serious threat to the quality and yield of apple, which lead to the export of apples was less than 3% of the total production. Different from other plant disease, apple virus disease lack effective control method at present[6,7]. The systematic research on the apple virus disease is great urgent for the quality and yield of apple.

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Apple virus disease is widespread all over the world[8,9]. Currently, 39 apple viruses have been reported, and 17 apple viruses have been identified in China[10]. All apple viruses are divided into latent and non-latent viruses, among which the latent viruses are mainly *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV)[11-14]. ACLSV is known to infect Apple, Peach, Pear, Hawthorn and Cherry causing major problems in the yield and quality of these fruit[15-18]. ACLSV maybe co-infection with other viruses, resulting in more serious decline of apple tree[19]. Therefore, systematically studying on the occurrence, damage and genetic variation of ACLSV is important to improve apple yield and quality.

In this study, 360 apple leaves were randomly collected in 12 cities (Linfen, Yunchengshiqu, Huozhou, Jixian, Linyi, Pingyao, Pinglu, Quwo, Taigu, Wanrong, Xiangfen, Xinjiang) of shanxi Province during 2015 and 2016. RT-PCR was used to detect and clone the ACLSV genomefrom these fresh leaves. The genetic Structure and molecular variation of ACLSV were further study, which may provide theoretical basis for the prevention of ACLSV.

Materials and Methods

Materials

The sample was collected from April to June in 2015 and 2016.A total of 12 sites in Shanxi Province were selected. The samples were randomly collected in each sites according to the diagonal direction. All the samples were transported to the laboratory within 24 h on ice box and marked the sample location and collection time.

Primer design and selection

The 25 complete genome sequence of ACLSV were obtained from NCBI (https://www.ncbi.nlm.nih.gov), and the prediction and analysis of conserved regions were performed by DNAMAN 6.0. Based on the genomic conserved region, 7 pairs of primers were designed by Premier. The 5′ and 3′ ends of the genome were amplified using the race kit (BioTeke, China).The primer sequences and expected amplification product sizes were shown in (Table 1).

Table 1 — The Primer sequences, size and products

Primer	Sequence (5′-3′)	Position	Product（bp）
5′race out primer	ACTGATAGAGAAAAGGGAGA	729-748	-
5′race inner primer	GCAAAAGGTGAGAAATAAAC	316-335	
F2	AAAACTCTGGAGAACCACCT	364-383	
R2	TGATCTGAACTTCGCCGCCT	1676-1695	1332
F3	TACTCTGATTTGCTGTCT	1567-1584	
R3	ATTTACACCTTTCTCGCT	3424-3441	1875
F4	GACGAAAAATGTGAAATGCT	3238-3257	
R4	TTTGCCTCTGTGAACCTCTT	4843-4862	1625
F5	TCAAAGAGTGCAGCCACGATA	4735-4755	
R5	TAGCACCATCCAGAAACACCA	6013-6033	1299
F6	ACTATATTCATTGGGGGGCTC	5955-5975	
R6	CCTTGATAAGATCCACTACCG	7003-7023	1069
3′race out primer	GACAAAATCAGACGAAGGAGG	6747-6767	-
3′race inner primer	CAACAAAATGACTTTCCGCCAG	7055-7076	

RNA extraction and RT-PCR

RNA was extracted from samples using the Universal Plant Total RNA Extraction Kit (BioTeke, China), and cDNA was synthesized using the Prime Script RT reagent Kit (BioTeke, China). PCR was carried out in a 25 μL PCR mixture including 2 μL of cDNA template, 2.5 μL of 25 mM Mg^{2+}, 2.5 μL of dNTP mixture with each dNTP at 5 mM, 2.5 μL of 10× polymerase buffer, 0.5 μL of 5 U/μL Hot-start Taq polymerase, and 2 μL sense and antisense primers (10 μM each). The reaction process was as follows: denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 52°C for 1 min, and primer extension at 72°C for 2 min; and final extension at 72°C for 10 min. For the 5′-terminal and 3′-terminal sequence, 5′ RACE and 3′ RACE reactions were conducted using the 5′ RACE and 3′ RACE system.

Sequencing genome assembly

The PCR products was examined by 2% agarose gel under UV light. The positive bands were purified from the agarose gel using a gel extraction kit (BioTeke, China). These fragments were inserted into a pGEM-T simple vector and cloned into *Escherichia coli* JM109. For each fragment, at least 3 clones from each ligation were sequenced. If there was any difference at any position of the sequences, at least 4 clones were sequenced to obtain the consensus sequence. All the positive sequence were used to assemble the genome of the ACLSV.

Phylogenetic and selection pressure analyses

All 25 ACLSV isolates were aligned using MEGA v.5.0[20]. The evolutionary relationships of these aligned sequences were determined by the maximum likelihood (ML), neighbour joining (NJ), and minimum evolution (ME) methods in MEGA v.5.0[21]. Bootstrap analyses with 1,000 replicates were performed to evaluate the significance of internal branches. Branches with < 50% bootstrap values were collapsed.

The selection pressure was estimated by the Ka/Ks ratio, where Ka represents the average number of non-synonymous substitutions per non-synonymous site, and Ks represents the average number of

synonymous substitutions per synonymous site. The values of Ka and Ks were estimated using the PBL method in DNAsp[22]. A gene was considered to be under positive (or diversifying) selection when the Ka/Ks ratio was >1, under neutral selection when the Ka/Ks ratio = 1, and under negative (or purifying) selection when the Ka/Ks ratio < 1[16].

Results

The incidence of ACLSV

RT-PCR results showed that the incidence of ACLSV was ranged from 43.59% to 68.18% (Fig. 1A). The highest incidence was found in Wanrong followed by Yunchengshiqu, Huozhou, and Taigu, and lowest in Linfen (Fig. 1B). General speaking, ACLSV distributed widely in Shanxi province, which has been a great threat to apple production. Since, Shanxi province was one of important apple production area, necessary measures should be taken to control ACLSV.

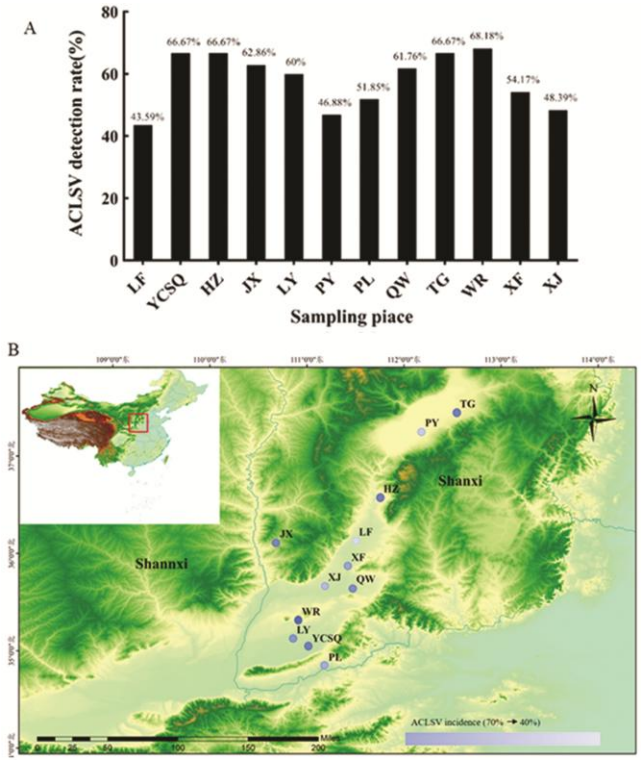


Fig.1 — The distribution of *Apple chlorotic leaf spot virus* (ACLSV) in shanxi provinces. (A) Incidence of ACLSV in different regions of Shanxi; and (B)The distribution of samples infected by the *Apple chlorotic leaf spot virus* (ACLSV) in shanxi provinces. Infected samples were widely distributed throughout 12 sites; the average detection rate for every site is shown in (Fig. A). (Fig. B) shows the difference in the average incidence in the 12 sites. The sites marked with blue color are where sampling was performed. The darker the shade of color was, the higher the incidence of viral infection. In the figure, LF stands for Linfen, YCSQ stands for Yunchengshiqu, HZ stands for Huozhou, JX stands for Jixian, LY stands for Linyi, PY stands for Pingyao, PL stands for Pinglu, QW stands for Quwo, TG stands for Taigu, WR stands for Wanrong, XF stands for Xiangfen, XJ stands for Xinjiang

ACLSV genome analysis

One new ACLSV isolate was got in this study. The complete genome of this new isolate including the 3′ and 5′ ends was 7507 bp and encodes a polyprotein of 2536 amino acids with a molecular weight of 610 kDa. The polyprotein was cleaved into three mature proteins (RNA polymerase, Movement protein, and Coat protein) (Fig. 2). ORF1 starting at ATG (139-141 bp) encoded RNA polymerase of 216 kDa, which was the largest protein; ORF2 encoded a movement protein (MP) of 50 kDa; ORF3 in the 3' end encoded a coat protein with 21 kDa. ORF1 and ORF2 shared 83 bp, which encoded the N-terminus of the MP and C-terminus POL. ORF2 and ORF3 shared 317 bp, which encoded the N-terminus of the CP and C-terminus MP.

The analyze on the nucleotide identity showed that the POL of the 25 isolates was ranged from 74.6% to 99.7% (Table 2), from 81.2% to 99.6%

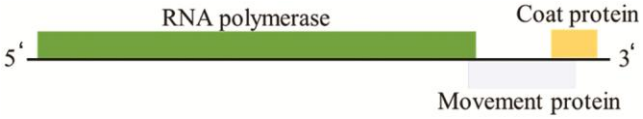


Fig. 2 — The bar presentation of ACLSV genome structure. The complete genome of ACLSV was 7507 bp including the 3′ and 5′ ends and contains a single large open reading frame (ORF) with 2536 amino acid. The putative ORF started at ATG (139-141 bp) in the sequence with an estimated molecular weight of 610 KDa. The polyprotein subsequently was cut into three proteins (RNA polymerase, Movement protein, Coat protein)

Table 2 — Nucleotide and amino acid identities of genes among twenty five ACLSV isolates

Gene	Interregional	Nucleotide Identity	Number of encoded amino acids	Amino acid Identity
RNA polymerase	139-5790	74.6%-99.7%	1884	81.9%-99.7%
Movement protein	5708-7084	81.2%-99.6%	435	81.2%-98.9%
Coat protein	6768-7349	83.5%-99.1%	194	75.8%-97.8%

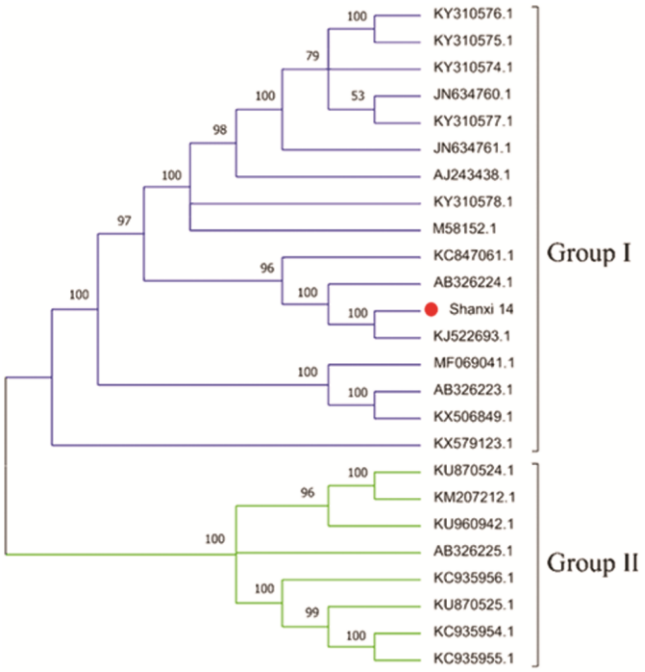


Fig. 3 — Maximum Likelihood tree based on the genome sequences of *Apple chlorotic leaf spot virus* (ACLSV). Bootstrap analysis was applied using 1000 replicates. Only bootstrap values (%) above 50 were given. Based on phylogenetic analysis of the complete genome sequence, 25 ACLSV isolates were clustered into two groups. The first group consisted of 17 isolates isolated from apples, peaches and cherries from different regions. The second group contained 8 isolates isolated from pears and hawthorn in different regions

for MP, and from 83.5% to 99.1% for CP. While in amino acid level, the identity was ranged from 81.9% to 99.7% for POL, and from 81.2% to 98.9% for MP, and from 75.7% to 97.8% for CP.

Genetic diversity of ACLSV

The phylogenetically of 25 ACLSV isolates were analyzed to understand the genetic diversity of ACLSV. The analysis showed that the 25 isolates were divided into two groups (Group I and II) (Fig. 3). Group I consisted of 17 isolates, which isolated from apples, peaches and cherries. The rest 8 isolates was belong to Group II, which was isolated from pears and hawthorn. The isolates from China were clustered into different groups, which confirmed that the genetic diversity of ACLSV isolates was not significantly associated with geographic location.

The complete genome identity among different isolates was also analyzed in nd level. For the new ACLSV isolate, the highest identity was

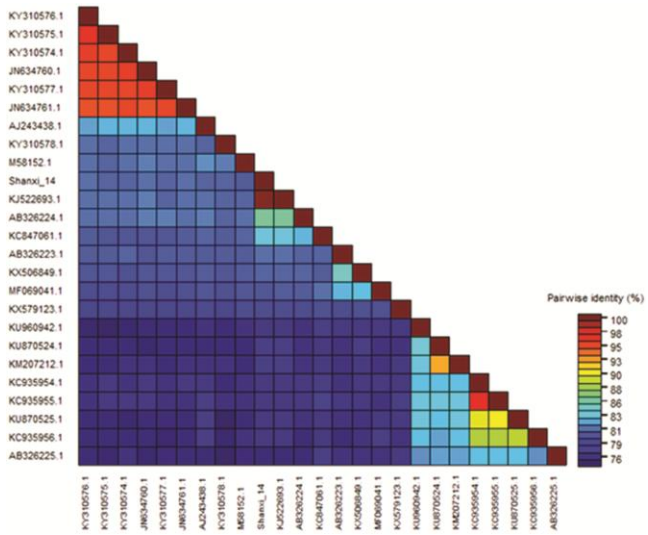
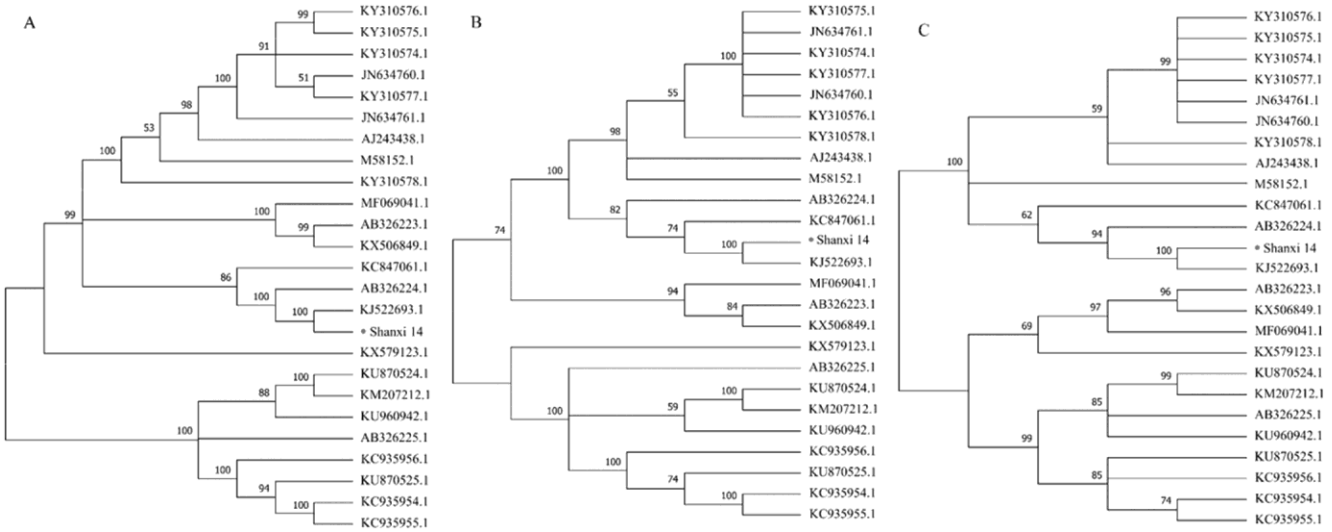


Fig. 4 — Two-dimensional distribution of the alignment of the nucleotide sequences of the ACLSV genome. The identity of the nucleotide sequences of the 25 isolates was calculated by SDTv software. Homology ranges from 70.4% to 99.8%, with red representing the highest homology of 100% and blue representing the lowest homology of 70%.According to the identity analysis of 25 isolates, the highest nd identity was between shanxi14 and KJ522693.1, and the lowest was shanxi14 and M58152.1

99.7% between shanxi14 and KJ522693.1 (Fig. 4), the lowest identity was 70.4% between shanxi14 and M58152.1%, which got the same result with phylogenetical analyze. The phylogenetic analyze of the 25 isolates was conducted by three functional domains (RNA polymerase, Movement protein, Coat protein) (Suppl. Fig. 1A-C) respectively, and the results showed that the phylogenetic tree based on POL were similar with whole genome. The genetic diversity level of POL may be more representative the whole genome. For CP gene, the higher variation was found in 3′ end than 5′ end. 3′ end of CP gene may be basis for the classification of ACLSV strains[23].

ACLSV selection pressure

The selection pressure on ACLSV isolates was analyzed (Table 3), and the Ka values for all the gene of all isolates were less than the Ks values, and the Ka/Ks ratio < 1. It was appeared that the genes of ACLSV were under negative selection pressure, which also confirmed by Tajima' D, Fu & Li' D (1993) and Fu & Li' F tests. For different genes, the Ka/Ks ratio was different, which showed the degree of selection pressure on different gene was diversity.



Suppl. Fig. 1 — Maximum Likelihood tree based on the three functional domains of *Apple chlorotic leaf spot virus* (ACLSV). Bootstrap analysis was applied using 1000 replicates. Only bootstrap values (%) above 50 are given. Based on phylogenetic analysis of the complete genome sequence, 25 ACLSV isolates were clustered into two groups, including field collection and reference sequences. (A) RNA polymerase segment; (B) movement protein segment; and (C) coat protein segment

Table 3 — Selection pressure of genes among twenty five ACLSV isolates

Gene	m	S	Ps	θ	π	Ks	Ka	ω	Tajima's D	Fu &Li'D	Fu &Li'F
RNA polymerase	25	2764	0.49	0.13	0.22	0.703	0.086	0.122	0.056	0.635	0.528
Movement protein	25	583	0.43	0.11	0.164	0.47	0.077	0.164	-0.104	0.287	0.189
Coat protein	25	203	0.35	0.09	0.129	0.252	0.094	0.373	-0.058	0.214	0.149
Complete genome	25	3425	0.478	0.13	0.209	0.594	0.104	0.175	0.035	0.579	0.475

Abbreviations: m= number of sequences; n=total number of sites; S=number of segregating sites; Ps=S/n; θ= Ps/a1; π=nucleotide diversity; Ks= Synonymous substitution; Ka= Nonsynonymous substitution; ω= Ka/Ks Mean (Ka/ Ks) values <1 indicates negative or purifying, mean (Ka/Ks) values = 1 suggests neutral selection, and mean (Ka/Ks) values > 1 indicates positive selection for each gene specific sequence data set; Tajima's D, Fu & Li'D and Fu & Li'F is neutrality tests

Discussion

ACLSV is great threat to apple production in Shanxi province. According to this study, the incidence of ACLSV was more than 50% in 9 sites, in which lead to great economic loss. In addition, the present results also showed that ACLSV could infect apple, and pear. These different isolates may co-infect leading to more serious economic losses[24,25]. Along with the development of apple industry, the exchange of apple germplasm more and more frequently, which also accelerated the spread of ACLSV in Shanxi province. Because RNA polymerases lack the proof-reading ability of DNA polymerases, ACLSV was therefore capable of very rapid evolution when faced with sufficient selective pressure, which improve difficulty to control ACLSV. Therefore, more attention must be paired to ACLSV to improve the apple yield and quality. Reasonable preventions and control strategies were necessary and important to prevent and control this virus.

ACLSV showed high genetic diversity. 25 ACLSV isolates from different areas were divided into two groups in this study, and the high variation was found in nd and aa level, respectively. In addition, the genetic diversity of ACLSV has a certain relationship with host since all pear and hawthorn isolates were cluster into Group II and the apples, peaches, prunus and cherries isolates were into Group I. Based on selection pressure analyze, the negative selection pressure maybe be an important factor for the genetic diversity of ACLSV. As RNA virus, ACLSV evaluate rapidly resulting in

many mutants. Host, environment and vector may generate selection pressure on ACLSV. The mutant who was adapt to the environment may generate the heritable variation improve the genetic diversity of ACLSV. The different style of fruit may be the important selection pressure on the evolution of ACLSV.

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