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Original Article

Molecular Detection of *Little Cherry Virus 1* (LChV-1) from Iran

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Abstract

Background and Aims: Although *little cherry disease* (LChD) is considered as one of the main virus diseases of cherry in several countries, however, to date, there is no data on the incidence of LChV-1 in Iran, the main region of commercial sweet cherry production in West Asia.

Materials and Methods: Sweet cherry orchards in north-west Iran including West Azarbaijan, East Azarbaijan Tehran and Qazvin provinces, were surveyed for *Little cherry virus 1* (LChV-1) infection. RT-PCR tests of symptomatic leaf samples, using specific primers for LChV-1 coat protein gene (CP). Using CP sequences the phylogenetic tree was constructed and molecular analysis and population differentiation was indicated by different approaches.

Results: Using specific primers a DNA fragment of the expected size about 500 bp in five out of 43 samples, were amplified. Phylogenetic analysis indicated that the LChV-1 isolates clustered into five groups, which three Iranian isolates fell into GII without a host or geography-based correlation. High nucleotide diversity was determined between the different phylogenetic groups, whereas the within-group diversity of LChV-1 CP gene was evens less. The low nucleotide diversity and high haplotype diversity indicated that the LChV-1 population experienced a bottleneck effect and the population underwent growth after a period of low efficient population size. In addition, the dNS/dS ratios indicate that the GIII is probably under weak positive selection and the GI and GII under strong negative selection.

Conclusions: This study showed for the first time the incidence of LChV-1 in sweet cherry orchards in Iran, which is putatively associated with various plant disorders .

Keywords: *Little cherry virus 1* (LChV-1), molecular detection, Iran

Introduction

Little cherry disease (LChD), has been reported on sweet cherry (*Prunus avium*), sour cherry (*P. cerasus*) and different ornamental cherry trees, which has a major effect on both fruit size and quality in the infected trees (1). Little cherry disease symptoms on fruit vary depending on host cultivar and mainly consist of small angular and pointed fruits, imperfectly colored, incompletely ripened and flavorless fruits in susceptible sweet cherry cultivars, whereas other cultivars show only moderate fruit deformations or are tolerant. In addition, fruit sweetness is reduced which results in lowering market value along with consumer acceptability (2). Two members of the *Closteroviridae* family, *Little cherry virus 1* (LChV-1), a member of the newly proposed genus *Velarivirus*, along with *Little cherry virus 2* (LChV-2) are associated with little cherry disease (LChD). Various isolates of LChV-1 have also been associated with recently reported disorders including Kwanzan stunting syndrome and Shirofugen stunt disease (3). At first, LChV-1 was considered to have a limited host range; however symptom-less LChV-1 infections have also been reported

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in other Prunus species including almond, peach, and plum (4, 5, 6).

The virus is graft-transmissible, but no known natural vector has been yet identified and its spread occurs through the movement of infected plant material.

Sweet cherry orchards are widely spread nearly across all geographical areas of Iran, covering a total area of 32,000 hectares with an annual production of 278,000 tonnes and 8.7 tonnes/ha yield (7). LChD is considered as one of the main virus diseases of cherry in several countries, however, to date, there is no data on the incidence of LChV-1 in Iran, the main region of commercial sweet cherry production in West Asia. In the previous studies, several virus diseases have been reported on stone fruit trees in Iran including *Arabis mosaic virus, Cherry leaf roll virus, Plum pox virus, Prunus necrotic ring spot virus, Prune dwarf virus, Raspberry latent ringspot virus, Tomato ringspot virus* (8, 9). In this study, we show the occurrence of LChV-1 in sweet cherry orchards in some major provinces of North West Iran, using a molecular detection method, RT-PCR, and nucleotide sequence analysis.

Methods

During 2017 different sweet cherry orchards in West Azarbaijan, East Azarbaijan, Tehran and Qazvin provinces were surveyed. Totally, 43 symptomatic leaf samples with mosaic, mottle, chlorosis, leaf deformation, and reduced growth and vigor were collected from sweet cherry trees.

Total RNA was extracted and purified using RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturers' instructions. Using specific primers designed by Katsiania et al. (10). The expected PCR products were excised from the gels and cleaned by the Wizard SV Gel and PCR Clean-Up kit (Pro-mega, USA). DNA sequencing was done in both directions.

Sequences of CP gene and predicted amino acid sequences of three Iranian LChV-1 isolates obtained in this study were compared with those of CP sequences in GenBank, including 30 isolates from Europe countries and two other isolates from China and South Korea. Phylogenetic analysis was inferred using the Neighbor Joining (NJ) method implemented in MEGA 5 (11). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary relationships of the taxa analyzed. In addition, the average nucleotide distance between the CP sequences was estimated using Kimura's two parameters implemented in phylip version 3.5 (12).

Mean nucleotide distance between two randomly selected sequence variants were estimated by the maximum-composite-likeli-hood method with MEGA version 4.02 (13).

Pairwise synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dNS) were also estimated using the PBL method based on Kimura's two-parameter model (14). Standard deviations were estimated by the bootstrap method, with 1000 repeats.

Furthermore, pairwise genetic were analyzed by the Kimura's two-parameter method implemented in using Phylip version 3.67 program (12). DNASP version 4.10 (15) was used to estimate haplotype diversity.

Results and Discussion

An amplicon of the expected size about 500 bp corresponding to coat protein (CP) gene of LChV-1 was obtained in five samples collected from West Azarbayja, Qazvin, and Tehran provinces. Five LChV-1 positive samples were also tested for the presence of other viruses including ArMV, CLRV, PNRSV, PDV, RspRSV, and ToRSV using ELISA method.

ArMV and PNRSV were detected separately in two samples. Reduced growth and chlorosis were the main symptoms associated with LChV-1 positive samples. There was no RT-PCR amplification with the total RNA obtained from healthy sweet cherry seedlings.

Using partial CP analysis LChV-1 isolates clustered into five distinct groups, which three Iranian isolates with German V2356 isolate clustered in group I (GI). Ponferrada, Taian, Jerte, and YD isolates grouped in GII and UW2 and ITMAR isolates clustered in distinct GIII (Fig. 1A). Two LChV-1 isolates Kyoto-2

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and G15 3 from Japan and Greece were diverged from other isolates and formed GIV and GV, respectively (Fig. 1A). Phylogenetic analysis using complete genome sequencing of different LChV1 isolates (3-5, 16, 17) indicated that UW2 and ITMAR isolates are highly similar and clustered with together, however the German isolate V2356 differs considerably from them. In addition, the isolates Ponferrada, Taian, Jerte, and YD formed a third phylogenetic group (18). Phylogenetic analysis of the different genes or complete genome shows no clear pattern of a host or geography-based grouping, which may be the result of world-wide transport of infected plant material (3).

Different molecular studies indicated genetic diversity among various LChV-1 isolates (18).

In this study the genetic distances between the different phylogenetic groups of LChV-1 are ranged from 0.0 to 40.3% based on CP nucleotide sequences (Fig. 1B). The lowest CP nucleotide diversity (0.0-13.9) was found for GI, followed by 13.9-27.8 for GII and 27.8-34.8 for GIII. Two Greek isolates G15 3 and C118-Iso1 were grouped in a separate branch (GIV), more distinct from other LChV-1 isolates with the highest nucleotide diversities (34.8-40.3) (Fig. 1B).

Haplotype diversity was estimated based on the frequency and number of haplotypes in the population. The mean nucleotide diversity for the CP gene was 0.208 ± 0.015 (Table 1).

In addition, the within-group diversity of LChV-1 CP gene was even less ranging from 0.024 ± 0.004 to 0.212 ± 0.017 (Table 1). The dNS/dS ratios differed for GIII in CP gene, in compre-ssion with GI and GII, which indicate that the GIII is probably under weak positive selection and the GI and GII under strong negative selection (Table1).

Positive, diversifying selection is an important evolutionary force, which accelerates the variation between homologous proteins (19).

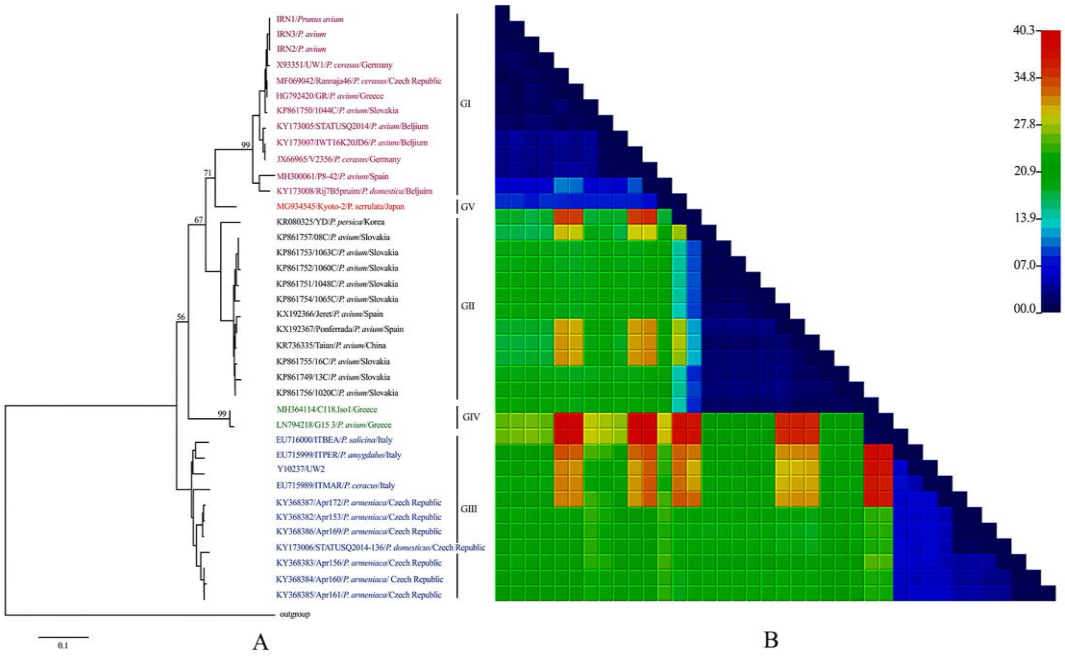

Fig. 1. (a) A Neighbor Joining (NJ) phylogenetic tree showing the relationship among *Little cherry virus 1* isolates. The tree was constructed from the coat protein nucleotide sequences of 38 LChV-1 isolates. Numbers at each node indicate the percentage of supporting puzzling steps (or bootstrap samples) in NJ method. The accession number in the international gene sequence data base (GenBank), name of each isolate, original host and its country of origin are listed. Phylogeneteic groups were colored by Pink, Black, Blue, Green and Red for GI, GII, GIII, GIV and GV, respectively. (B) Two dimensional percentage nucleotide diversity plot of the coat protein gene of 38 LChV-1 isolates.

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Table 1. Nucleotide and haplotype diversity of CP gene of *Little cherry virus 1* (LChV-1) isolates[a]

Group	Db	Haplotype diversity	d_S	d_{NS}	d_{NS}/d_S
GI	0.024 (0.004)	0.987	0.083 (0.015)	0.004 (0.002)	0.048
GII	0.028 (0.005)	0.963	0.087 (0.019)	0.004 (0.002)	0.045
GIII	0.212 (0.017)	0.981	0.375 (0.019)	0.081 (0.039)	0.216
All isolates	0.205 (0.015)	0.993	0.908 (0.055)	0.032 (0.011)	0.035

[a] Substitutions: *dNS* = average number of nonsynonymous substitutions per nonsynonymous site, *dS* = average number of synonymous substitutions per synonymous site, and *dNS/dS* = average of the ratio between nonsynonymous and synonymous substitutions.
[b] *D* = nucleotide diversity: average number of nucleotide substitutions per site between all pairs of sequences in the group. Standard errors are indicated in parentheses.

In many proteins, most amino acid residues are highly conserved as a result of structural-functional necessity, so, positive selection most probably influence only a limited number of sites. Coat proteins have multifunctional duties in the life cycle of plant viruses. Therefore, negative selection constrained on CP gene of LChV-1 indicated that variation in this gene may affect the CP structure, fitness or completely abolishes LChV-1 infectivity.

The haplotype diversity and the nucleotide diversity for all isolates were 0.993 and 0.205, respectively (Table 1). The low nucleotide diversity and high haplotype diversity (Hd > 0.5) indicated that the LChV-1 population experienced a bottleneck effect and the population underwent growth after a period of low efficient population size (20).

On the other hand, lower nucleotide diversity and high haplotype diversity values illustrated that the time after population development is sufficient long to examine the variation in haplotypes that arising from mutation, but it is not sufficient long to accumulate high diversities between sequences (21).

This study showed for the first time the incidence of LChV-1 in sweet cherry orchards in Iran, which is putatively associated with various plant disorders.

The precise descriptions of phylogenetic relationships and comparisons between individually related virus genomes provide a valuable approach for understanding of main evolutionary mechanisms.

In addition, analysis of this diversification is important for improv-ing advances in different control programs of viral diseases in order to prevent their distri-bution.

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