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Molecular Characterization of *Peach Latent Mosaic Viroid* Variants Isolated From Stone Fruit Trees in Kurdistan Province, Iran

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ABSTRACT

In order to investigate the possible presence and molecular features of *Peach Latent Mosaic Viroid* (PLMVd) in west Iran (Kurdistan Province), a total of 132 leaf samples from almond, apricot, nectarine, peach, plum, sour cherry, and sweet cherry were collected from orchards during the summer of 2016 and 2017. Reverse transcription-polymerase chain reaction amplified an expected ~350 base pair DNA fragment from 34 samples. The complete genome sequencing of 17 cloned isolates was determined. Sequence alignment of the new sequences showed 94.3-100% nucleotide identity, and 79.2-100% nucleotide identity with other previously reported *PLMVd* isolates. In phylogenetic analysis, isolated viroid variants from this study and 32 previously reported isolates were placed in two groups (I and II). All the isolated viroid variants in the present study were placed in group II-A (mosaic-inducing isolates), together with other isolates from Australia, China, India, Iran, Spain, Tunisia, and Turkey. The secondary structure of the Iranian variants revealed their unique structures as compared with previously reported isolates of the viroid. To our knowledge, this is the first report of *PLMVd* infection on apricot, sweet cherry, sour cherry, and nectarine in Iran.

Keywords: Phylogenetic analysis, *PLMVd* hosts, Secondary structure, Sequence alignment.

INTRODUCTION

Up to now, 32 viroid species have been identified, which are classified into two families: *Pospiviroidae* and *Ausunviroidae* (Di Serio *et al*., 2014). In contrast to *Pospiviroidae*, members of *Avsunviroidae* do not possess a Central Conserved Region (CCR) and they are able to catalyze self-cleavage of multimers produced during symmetric rolling-circle replication mechanism (Flores *et al*., 2017).

The family Avsunviroidae includes three genera: Avsunviroid with the mono species Avocado sunblotch viroid, Pelamoviroid with two species *Chrysanthemum chlorotic mottle viroid* and *Peach latent mosaic viroid*, and Elaviroid with the species Eggplant latent viroid (Di Serio *et al*., 2014). Peach Latent Mosaic Viroid (PLMVd) is circular single-stranded RNA molecule comprising 335-351 nucleotides (nt) and high degree of base pairing, possesses a branched secondary structure containing 11 stems (P1-P11), 11 loops (L1-L11), and one or more pseudoknots (Bussie`re *et al*., 2000; Pelchat *et al*., 2000; Fekih Hassan *et al*., 2007). PLMVd has hammerhead conserved domains in both polarity strand that serve for self-cleavage during replication (Flores *et al*., 1992).

PLMVd is the causal agent of Peach latent mosaic (PLM) disease, which was reported for the first time in France when the peach "GF-305" indicator graft was indexed by new peach cultivars imported from the USA and Japan (Desvignes, 1976). Most of *PLMVd*

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variants are latent and the infected trees remain non-symptomatic for several years and then develop different leaf symptoms including mosaic, chlorosis, blotching, vein banding, severe chlorosis or albinism called Peach Calico (PC) disease. Tree species of the family *Rosaceae* including apple, almond, apricot, peach, sweet cherry, plum, quince, and cultivated and wild pear have been considered as hosts for *PLMVd* (Kyriakopoulou *et al*., 2017). *PLMVd* can be transmitted by pollen (Barba *et al*., 2007), grafting and budding (Desvignes, 1986; Hadidi *et al*., 1997), but not by seeds (Howell *et al*., 1998). *PLMVd* was reported to be transmitted by aphids under greenhouse condition and still less is known about its epidemiology (Desvignes, 1986; Flores *et al*., 1992).

Boubourakas *et al*. (2011) pointed out that the titer of *PLMVd* in non-peach hosts was about 0.4% of that in cultivated peach by quantitative real time PCR tests. Also, plant species outside the *Rosaceae*, such as grapevine and mango, have been reported as hosts of *PLMVd* by tissue print hybridization (El-Dougdoug *et al*., 2012).

The genome size of *PLMVd* isolates from peach is varied and consists of 335 to 351 nt (Ambros *et al*., 1998; Fekih Hassan *et al*., 2007; Malfitano *et al*., 2003) and the nucleotide range from other trees are about 337-340 nt (Kyriakopoulou *et al*., 2017). *PLMVd* shows high natural polymorphism (Pelchat *et al*., 2000; Fekih Hassan *et al*., 2007) and replicates in its host based on quasi-species manner. Analysis of the progenies of a single *PLMVd* isolate revealed extreme variation of this viroid, which might be due to high mutation rate (Ambros *et al*., 1999; Loreti *et al*., 1999; Glouzon *et al*., 2014). Such heterogeneity property causes difficulties in the recognition of the relationship between any individual genotype and phenotype. However, PC symptom has been shown to be incited by the *PLMVd* variants that contain additional 12-13 nt insertion at the end of *PLMVd* genome along with a "UUUU" core in L11 between position 1 and 337 in the predicted secondary structure of *PLMVd* RNA (Flores *et al*., 2017); Mavric Plesko *et al*., 2012). Also, in another study, it has been shown that residue U at position 338 is responsible for the yellowish symptom and C at position 338 is responsible for the chlorosis-edge symptom (Wang *et al*., 2013).

PLMVd occurs in most commercial peach varieties worldwide and its occurrence in Iran was reported seven years ago just in one study. Yazarlou *et al*. (2012) tested 100 peach trees and 10 plum trees in northeastern Iran by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and compared them with Australian isolates. The authors found that 34 peach trees and 5 plum trees were positive for *PLMVd*. They also showed that the infectious clone generated two progeny viroid molecules with 10 different mutations compared with the parent clone inoculated 30 days before. *PLMVd* is on the list of the diseases for quarantine in Iran and there is lack of information about the presence of this viroid in fruit trees other than peach and plum.

In the present work, we aimed to analyze some stone fruit trees from several locations in west Iran (Kurdistan Province) to evaluate the incidence and genetic diversity of new sequences of Iranian variants of *PLMVd*.

MATERIALS AND METHODS

Sample Collection

During July, August, and September of 2016 and 2017, leaf samples from 132 stone fruit trees including almond, apricot, nectarine, peach, plum, sour cherry and sweet cherry showing viroid-like symptoms were collected from several locations of Kurdistan Province, Iran.

Total nucleic acid extraction

Total Nucleic Acid (TNA) was extracted from each leaf sample as described by Foissac *et al*. (2000) with minor

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modifications. At first, 100 mg of leaf tissue were homogenized in a mortar with 1 mL of grinding buffer (4.0 M guanidine thiocyanate, 0.2M NaOAc, 25 mM EDTA, 1.0M KOAc, 2.5% w/v Polyvinylpyrrolidone (PVP-40), 1% β-mercaptoethanol), and centrifuged at 900 g for 4 minutes. Then, 500 µL of supernatant were mixed with 100 µL of 10% (w/v) sodium N-lauroylsarcosyl solution, and incubated for 10 minutes at 70˚C, with intermittent shaking. After centrifugation at 12,500×g for 5 minutes, 300 µL of the supernatant were mixed with 1 g mL^{-1} silica suspension at pH 2, 300 µL of 6M NaI and 150 µL of 95% ethanol, and incubated for 10 min at room temperature with intermittent shaking. Silica was pelleted and washed twice with washing buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, and 50% ethanol). Finally, TNA was eluted by incubation for 4 min at 70°C with 70 µL of RNase-free water.

RT-PCR

RT reactions were performed in 10 µL final volume containing 1µL (0.2 µg mL^{-1}) random hexamer primer, 4 µL aliquot of extracted total RNA and 5 µL cDNA synthesis solution from the kit (HyperScriptTM Reverse Transcriptase, GeneAll, Seoul, Korea). The mixture was incubated at 55ºC for 60 minutes in a BioRad thermocycler (BioRad, USA). PCR reactions were done using PCR master mix (Tris-HCl pH 8.5, $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 U μL^{-1} Ampliqon *Taq* DNA polymerase) (GeneAll, Seoul, Korea). The PCR reaction contained 1X Kapa buffer A (+Mg), 0.4 µM RF43 and RF44 primers (Ambros *et al*., 1998) 0.2 µM dNTPs, 1X cresol loading dye (20% w/v sucrose, 1 mM cresol red), 4.75 µl RNase-free sterile distilled water and 1 µL cDNA in a 12.5 µLl reaction. The cycling conditions of the PCR were 94°C for 2 minutes for the initial denaturation, 35 cycles of 94°C for 30 seconds, annealing temperature for 30 seconds and 72°C for 1 minute for the extension followed by a final elongation step for 7 minutes at 72°C. PCR products were electrophoresed on a 1.2% (w/v) agarose gel (containing 0.5 µg μL^{-1} EtBr) in 1X TAE buffer. Electrophoresis was carried out at 80 V for approximately 1 h. Fragments were visualized with a UV transilluminator and photographed in a gel documentation (UVP, USA) apparatus.

Cloning, Sequencing and Sequence Analysis

To verify the PCR results, amplified fragments of eighteen isolates were ligated into the pTG19 vector (SinaClon, Iran) by T4 DNA ligase (SinaClon, Iran) according to the manufacturer's instruction with the minor modification using 25 ng of the vector and about 10 ng of the PCR product. The ligation mixture was incubated at 16ºC for 1 hour and then at 22ºC overnight as instructed by the manufacturer. The recombinant vector was introduced into competent *Escherichia coli* strain DH5α cells and the transformed cells were selected on LB plate containing ampicillin (100 µg mL^{-1}), IPTG (50 µL of 0.1M per plate) and X-Gal (10 µL of 50 mg mL^{-1}) as described elsewhere (Sambrook *et al*., 1989). Three white colonies for each *PLMVd* isolate growing on the LB selection plate were grown in 5 mL LB medium, and kept overnight at 37ºC before subjecting to plasmid purification by the alkaline lysis method (Ish-Horowicz and Burke, 1981) or using Exprep plasmid SV (GeneAll, Seoul, Korea). Purified plasmids were digested with *Bam*HI restriction enzyme, followed by electrophoresis on 1.5% agarose. Each colony carrying the cloned cDNA was isolated, purified and sequenced with T7promoter universal primers by Macrogen Inc. (Seoul, South Korea) and compared to GenBank isolates using the BLASTn program (Altschul *et al*., 1990).

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Phylogenetic, Genetic Diversity Parameters, and Secondary Structure

Neighbor joining (NJ) phylogenetic trees were constructed using the MEGA7 program (Kumar *et al*., 2016) with 1,000 replications to assess node support. The program DnaSP version 6.10.01 (Rozas *et al*., 2017) was used to assess genetic differentiation and estimate number of Haplotypes (H), Haplotype diversity (Hd), number of polymorphic (Segregation) sites (S), the overall average number of differences, k, and total number of mutations η (Eta). The predicted secondary structure of minimal free energy of the new Iranian *PLMVd* variants from stone fruits were obtained with the MFold program for circular molecules (Zuker, 1989).

RESULTS

Detection of PLMVd by RT-PCR

During survey of stone fruits in Kurdistan Province, 132 trees from seven different geographic areas were tested by RT-PCR for the presence of *PLMVd* infection. This viroid was detected in 34 samples in stone fruits. *PLMVd* was detected in 1 out of 5 samples (20%) of almond, 4 out of 29 samples (13.8%) of apricot, 5 out of 8 samples (62.5%) of nectarine, 10 out of 37 samples (27%) of peach, 6 out of 22 samples (27.3%) of plum, 1 out of 10 samples (10%) of sour cherry, and 7 out of 21 samples (33.3%) of sweet cherry (Table 1). No amplifications were obtained from healthy and water controls. Sporadic symptoms observed in stone fruit trees were not consistently associated with the presence of *PLMVd*.

Molecular Characterization of the Stone Fruits Isolates of PLMVd

For each of the *PLMVd* isolates, one complete cDNA clone was sequenced. Nucleotide sequence analysis of cloned *PLMVd* from stone fruits plants indicated that the apricot isolates were 339 nt long, sweet cherry, sour cherry and nectarine isolates 337 and 339 nt long, peach isolates 338 to 340 nt long, and plum isolates 339 and 340 nt long (Table 2).

To estimate genetic diversity of the *PLMVd* sequences based on full-genome sequence, several genetic diversity parameters were calculated (Table 3). The nucleotide diversity (π, 0.029), the Daplotype diversity (Hd, 0.993), the average number of differences (k, 10 nt), the number of Segregation sites (S, 38 sites) and mutations within the segregating sites (η, 42) were found in the new Iranian *PLMVd* isolates.

Sequence alignment of new Iranian *PLMVd* sequences with all the *PLMVd* variants in databases revealed that the 16 out of 17 sequences were novel variants. The observed substitutions, insertions, and deletions were unevenly distributed along the *PLMVd* molecule. Mutational analysis

Table 1. Results of RT-PCR for *PLMVd* detection in stone fruits from different regions of Kurdistan Province, Iran.

Sampling area	Almond	Sour cherry	Sweet cherry	Plum	Apricot	Nectarine	Peach	Total
Sanandaj	1/3	0/5	2/9	0/8	1/16	1/2	4/19	9/62
Marivan	-	0/1	0/1	0/2	0/1	0/1	1/2	1/8
Saqqez	-	-	1/3	0/1	0/3	-	0/2	1/9
Baneh	0/2	0/2	0/1	2/4	1/2	3/3	3/6	9/20
Kamyaran	-	1/2	2/4	1/3	1/2	1/2	1/7	7/20
Dehgolan	-	-	2/2	3/3	1/3	-	1/1	7/9
Qorveh	-	-	0/1	0/1	0/2	-	-	0/4
Total	1/5	1/10	7/21	6/22	4/29	5/8	10/37	34/132

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Table 2. List of samples collected, leave symptom observed, sequence analysis and accession numbers.

Isolate	Host	Symptoms[a]	Length (nt)	Acc Nos
Ba8	Sour cherry	N	337	KU903003
BH5	Peach	M	340	MF574156
BSh4	Nectarine	M	337	MF574158
BSh9	Nectarine	M	339	MF574159
D1	Apricot	N	339	MG788240
D2	Sweet cherry	NS	339	MG788241
D4	Plum	NS	340	MG788242
D6	Peach	M	338	MG788244
K2	Plum	N	339	MG788245
K3	Sour cherry	NS	339	MG788243
K5	Sweet cherry	M	339	MG788246
K6	Apricot	CS	339	MG788247
K9	Sweet cherry	CS, NS	339	MG788248
KH10	Peach	LD, M	338	MF574157
MH35	Peach	LD, M	339	MF574160
S2	Peach	YM	339	MG788249
S1	Nectarine	YM	339	KU903007

[a] N: Non-symptomatic; M: Mosaic; NS: Necrotic Spot; CS: Chlorotic Spot; LD: Deformation, YM: Yellow Mosaic.

Table 3 Genetic analysis of complete genome of *PLMVd* from different stone fruit trees.[a]

Sequences	Π	N	H	Hd	S	η	K
Sequences from this study	0.029	17	16	0.993	38	42	9.772
GenBank sequences	0.106	23	23	1.000	102	117	33.455
All sequences	0.084	40	38	0.997	106	125	26.049

[a] N: Sample size; H: Number of Haplotypes/isolates; S: Number of polymorphic (Segregating) sites; η (Eta): Total number of mutations; k: Average number of nucleotide differences between sequences; π: Nucleotide diversity.

showed that most of the variations were located in the regions spanning nucleotides 340 to 68 and 101 to 150 of the alignment (Figure 1). Figure 1 also shows the primary structure of the 17 Iranian *PLMVd* RNAs characterized in this study aligned with respect to the *PLMVd* reference sequence (Hernandez and Flores, 1992). Of a total of 341 nucleotide positions in the alignment, 29 positions (8.5%) were polymorphic for isolate K5; 28 positions (8.2%) were polymorphic for isolates D1, D6, K6 and K9; 27 positions (7.9%) were polymorphic for isolates S1, and D2; 26 positions (7.6%) were polymorphic for isolates S2, and BSh9; 25 positions (7.3%) were polymorphic for isolates K2, and K3; 24 positions (7%) were polymorphic for isolate BH5; 22 positions (6.4%) were polymorphic for isolates BSh4, and MH35; 20 positions (5.9%) were polymorphic for isolates KH10, and, D4, and 19 positions (5.6%) were polymorphic for isolate Ba8. Collectively, 42 (12.3%) polymorphic positions were identified in the *PLMVd* variants characterized in this study.

Phylogenetic Analysis

In phylogenetic tree, the 49 isolates were divided into two major phylogenetic groups: Groups I and II, with two subgroups in each group (Figure 2). Of these, eight isolates from Canada, South Korea, Turkey, China, Spain and Tunisia were placed in Group I. Other *PLMVd* isolates including all 17 isolates sequenced in this study, six isolates from Iran, three isolates from Greece, three isolates from

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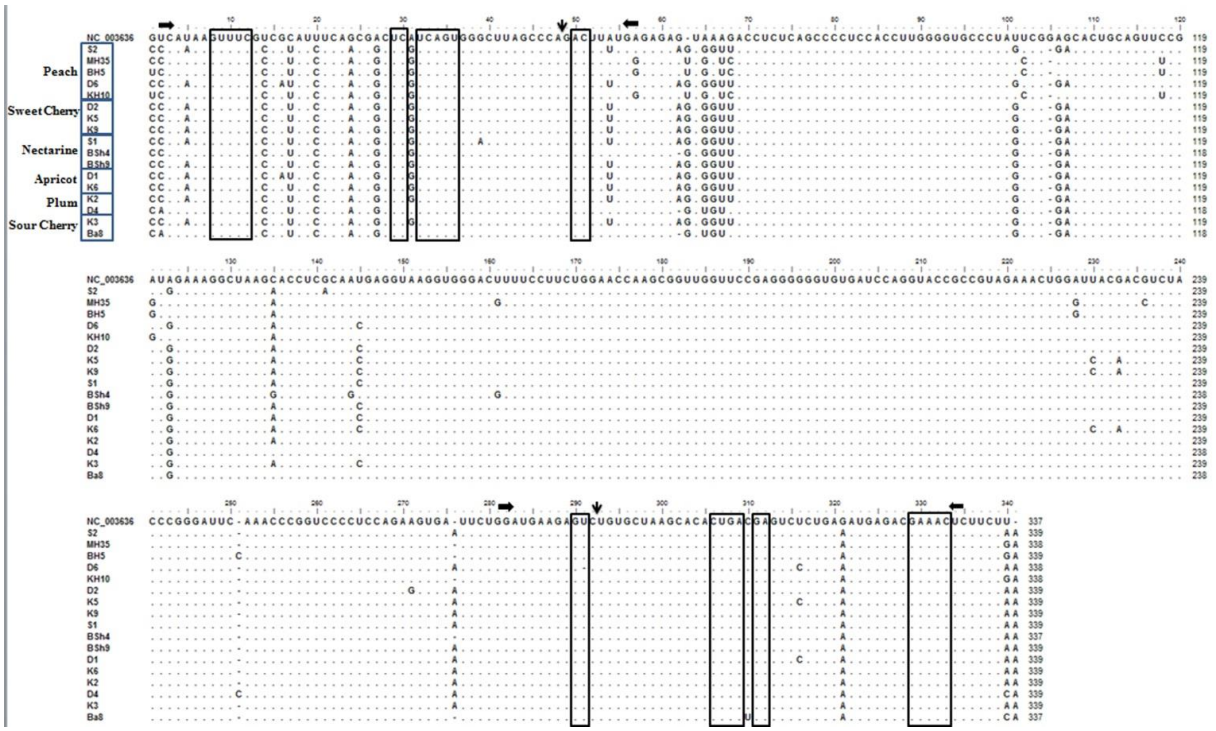

Figure 1. Sequence alignment of the 17 molecular sequences of *PLMVd* derived from stone fruit trees of Kurdistan Province, Iran. For comparative purposes, the reference sequence of *PLMVd* (Acc. No. NC_003636) is included at the top with two corrections, a deletion of one of the three Cs at positions 117 to 119 and a duplication of the G at position 257, with respect to that published previously (Hernandez and Flores, 1992). Dots indicate residues identical to the reference sequence, and dashes denote gaps. Regions involved in forming plus- and minus-polarity hammerhead structures are flanked by horizontal arrows, the conserved nucleotides present in most natural hammerhead structures are indicated on a box, and the self-cleavage sites are shown by vertical arrows. Primers used for RT-PCR amplification cover positions 178 to 225.

Turkey, two isolates from Australia, two isolates from Spain, two isolates from Italy, two isolates from India and one isolate from, China, South Korea, the USA and Tunisia were in Group II. Genetic mean distance within Group I isolates was 0.066±0.008, while this ratio was 0.055±0.007 within Group II isolates.

Secondary Structure of Iranian *PLMVd* Isolates

Secondary structure revealed the highest thermodynamic stability (i.e. the lowest Gibbs free energy, ΔG) and nucleotide co-variation analysis. Excluding the P8 stem, in all variants the P1, P2, P3, P4, P5, P6, P7, P9, P10, and P11 stems appeared in the most stable structures. As the P8 stem sequences were covered by RF-44 primer, nucleotide changes in this region were not detected. Variations appeared predominantly in the regions including the P1, P3, P7, and P11 stems, as opposed to those forming the P2, P6, and P9 stems (Figure 3).

We found frequent nucleotide co-variations in the new Iranian *PLMVd* variants for the formation of a pseudoknot by the nucleotides of the positions 339 to 2 in the loop 11 with those of the positions 64 to 67 in the L1 and L11 loops (Figure 4). Also, a similar interaction was obtained with the minus-polarity *PLMVd* strand (data not shown).

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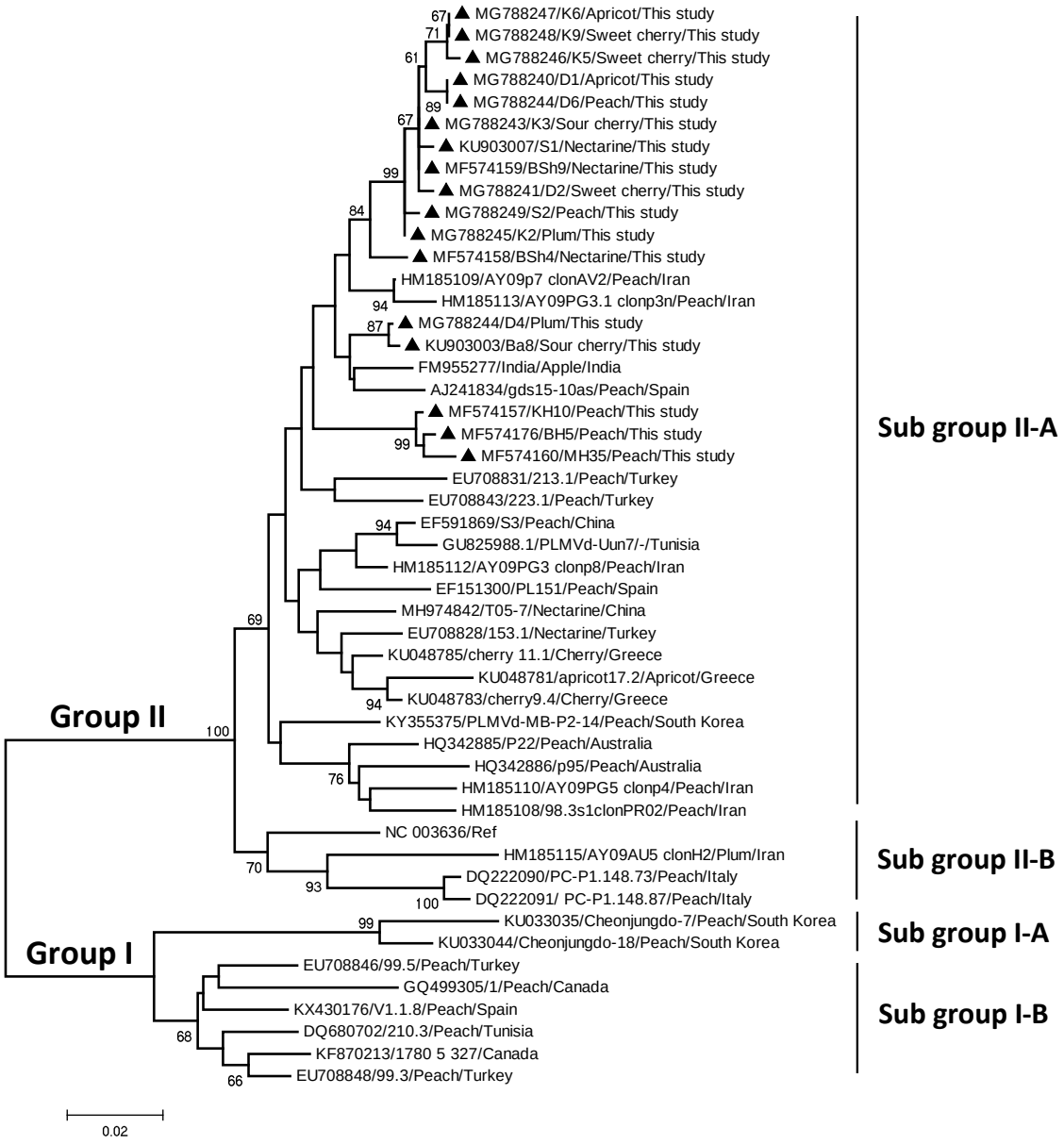

Figure 2. Unrooted phylogenetic tree of *PLMVd* isolates based on a multiple alignment using Clustal W and the neighbour-joining method within the MEGA7 program between the 17 *PLMVd* sequences characterized in this study and 32 selected *PLMVd* isolates from different hosts and countries (with GenBank accession number, host, and country used for this study). New Iranian isolates are shown by ▲mark.

DISCUSSION

A study on the identification and characterization of *PLMVd* isolates from several stone fruit trees in west of Iran was performed by more sensitive RT-PCR method using RF43 and RF44 primers. The reason for using these primers was sequence conservation in the P6 and P7 stems of the Iranian isolates in the previous study by Yazarlou *et al*. (2012). Although in a study (Serra *et al*., 2017), multiple changes in the P6 stem delimited by positions 161–197 (corresponding to the 5´ part of RF43 primer) were identified in two isolates, V1 and V2, these primers were used extensively

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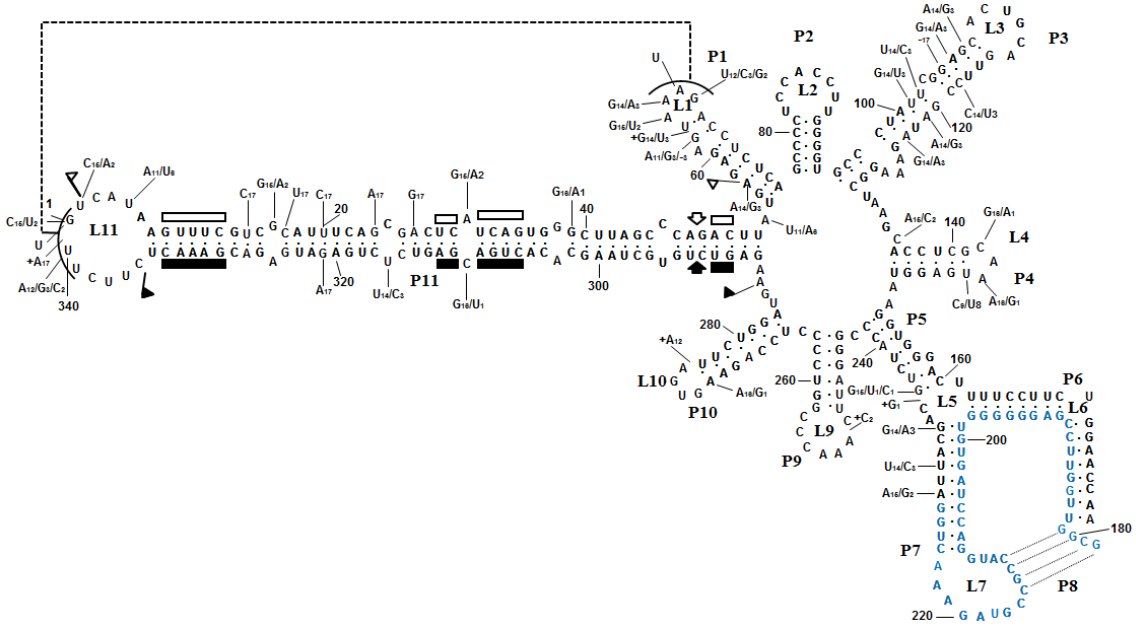

Figure 3. Primary and predicted secondary structure of lowest free energy of the reference variant of *PLMVd* RNA (Hernandez and Flores 1992; Ambros *et al*., 1998) with the mutations of the 17 new Iranian sequences. Plus and minus self-cleaving domains are delimited by flags, residues conserved in most natural hammerhead structures are indicated by bars, and the self-cleavage sites are indicated by arrows. Open and solid symbols refer to plus and minus polarities, respectively. Residues involved in a pseudoknot between positions 178–181 and 211–214, proposed on the basis of *in vitro* mapping assays with nucleases (Desvignes, 1986), are indicated by broken lines. Numbers in subscript refer to independent isolates containing each mutation. The position of the used primers in PCR (RF43 and RF44 (Ambros *et al*., 1998)) is shown by blue colour.

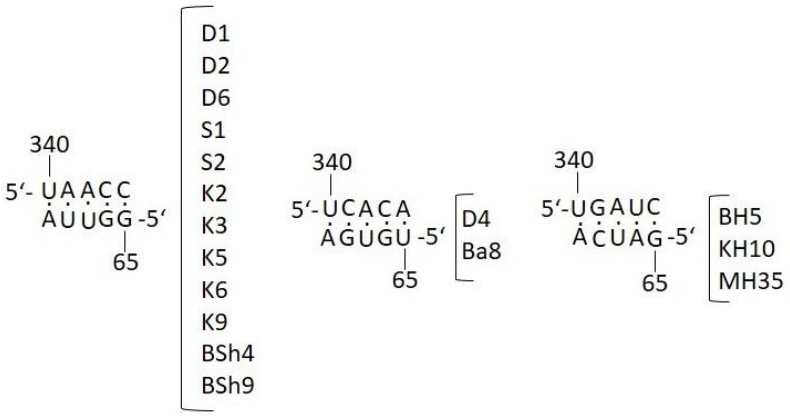

Figure 4. Potential pseudoknot interactions between nucleotides of the L11 and L1 loops. The *PLMVd* isolates/variants presenting this pseudoknot are shown in bracket.

in the characterization of *PLMVd* isolates and progeny variants (Ambros *et al*., 1999; Yazarlou *et al*., 2012). Besides, the P7 stem (corresponding to the RF44 primer) appeared to be more conserved (Glouzon *et al*., 2014) and suited to this purpose. *PLMVd* detection by RT-PCR in apricot, nectarine, peach, plum, sweet cherry, and sour cherry trees was also confirmed using a second pair of primers, C1/C2 (Loreti *et al*., 1999).

PLMVd was detected in 25.7% of collected samples by RT-PCR (Table 1). In

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comparison with other countries, these results showed that stone fruit trees grown in commercial orchards in west of Iran are not frequently infected by this viroid. The infection level of *PLMVd* in other countries is relatively high, i.e. 82% in Spain (Badenes and Llacer, 1998); 70% in Italy (Faggioli and Barba, 2006); 63-80% in different regions of Tunisia (Fekih Hassan *et al*., 2007); 62% in Syria (Ismaeli *et al*., 2001); 52% in Albania (Torres *et al*., 2004); 50% in the USA (Skrzeczkowski *et al*., 1996); and 40% in Montenegro (Mavric Plesko *et al*., 2012). However, a low incidence of *PLMVd* was also reported in eastern part of Turkey (3%) (Sipahioglu *et al*., 2006), in western Anatolia (17%) (Gumus *et al*., 2007) and in the eastern Mediterranean Region (19.6%) (Gazel *et al*., 2008). Yazarlou *et al*. (2012) reported the presence of the viroid in peach and plum trees in east and north of Iran with an incidence of 34 and 50%, respectively. Two possibilities may explain the low incidence of *PLMVd* in Iran. First, *PLMVd* is on the list of pathogens for quarantine in Iran but as this viroid can be latent for a long time in the plant nurseries (Flores *et al*., 2006), the disease can be imported by non-tested and apparently healthy material as rootstocks or grafted scions. Second, due to traditional agriculture in Iran, most gardeners use their own rootstocks or grafted scions and, therefore, the possibility of the viroid infection by imported infected-material would be decreased. Indeed, to limit further dissemination of *PLMVd* in a country, implementation of large-scale certification program is necessary.

RT-PCR products of *PLMVd* amplification were cloned and sequenced. A total of 17 sequences were obtained. We observed no sequence variant–host correlation and no regional specificity (Figure 1). For example, D6 sequence was obtained from peach tree but most closely related to D1 isolated from apricot (genetic distance: 0.003±0.003) in comparison to other variants isolated form peach trees (genetic distances: S2 (0.015±0.006), BH5 and MH35 (0.065±0.013), and KH10 (0.057±0.012). Also, the isolates K6 and K9 from apricot and sweet cheery, respectively (Table 2), had 100% identity. In the same way, we found no sequence basis for distinguishing *PLMVd* variants isolated from apricot, nectarine, peach, plum, sweet cherry, and sour cherry trees, which indicated that there was no host-related sequence specificity. This circumstance was already reported for *PLMVd* variants isolated from peach, pear, and almond (Fekih Hassan *et al*., 2007).

In agreement with previous studies (Bussie`re *et al*., 2000; Gumus *et al*., 2007; Hadidi *et al*., 1997), *PLMVd* variants were placed in two groups (I and II) and the new Iranian variants were clustered in Group II (Figure 2), similar to that of typical yellow mosaic-inducing *PLMVd* variants in peach (Malfitano *et al*., 2003). Variants of Groups I and II had differences in several positions in the *PLMVd* sequence alignment (Table 4) including 168, 170, 172, 174-176, 187-189, 198, 267-268, 274, 279, 284, 291-292, 330-331, and 339-340. Also, in the subgroups of Group II, distinct differences were observed between subgroup II-A and II-B (PC-inducing symptom) in positions 17, 20, 24, and 106. Note that some variants of subgroup II-B containing additional 12-13 nt insertion in the end of *PLMVd* genome and this part of the genome is responsible for inducing PC symptoms (Malfitano *et al*., 2003) (Table 4). In Group I, subgroups I-A and I-B showed several differences in positions 177, 190-193, 204, 208-209, 227, 242-243, 247, 258, 262, and 266 (Table 4).

Since stone fruit trees are naturally infected by *PLMVd*, a broad range of sequence heterogeneity should be expected. Variations appear predominantly in the regions including the P1, P3, P7, and P11 stems, as opposed to those forming the P2, P6, and P9 stems (Figure 3). In the new Iranian sequences, P3 was very variable while this motif in pervious study (Yazarlou *et al*., 2012) showed less variation. Based on the sequence variation and secondary structure, *PLMVd* comprises two domains

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Table 4. Positions in *PLMVd* alignment with their nucleotide differences in 2 groups and 4 subgroups.

Phylogenetic group	Positions in alignment													
	17	20	24	106	168	170	172	174-176	177	187-189	190-193	198	204	208-209
II-A	U	C	A	G	U	G	G	AAC	C	GUU	CCGA	G	G	CA
II-B	C	U	G	A	U	G	G	AAC	C	GUU	CCGA	G	G	CA
I-A	C	C	G	A	C	C	C	GUU	C	AAC	UAGA	A	A	UU
I-B	C	C	G	A	C	C	C	GUU	U	AAC	CCAG	A	G	CA

Phylogenetic group	Positions in alignment													
	227	242-243	247	258	262	266	267-268	274	279	284	291-292	330-331	339	340
II-A	U	AC	G	C	U	C	UC	A	A	G	GA	AG	U	C
II-B	U	AC	G	C	U	C	UC	A	A	G	GA	AG	U	C
I-A	A	UA	A	U	C	U	CU	G	C	A	AG	GA	C	U
I-B	U	AC	G	C	U	C	CU	G	C	A	AG	GA	C	U

(Pelchat *et al*., 2000; Fekih Hassan *et al*., 2007): a sequence variation with conserved secondary structure (left domain), and a relatively conserved sequence (right domain). The main difference between the new Iranian variants and previously studied variants (Pelchat *et al*., 2000) concerns stem P3. In the former, this stem shows low variability, but in the new Iranian variants it shows a high level of nucleotide variability. Moreover, in agreement with the Tunisian variants (Fekih Hassan *et al*., 2007) and in contrast to the other previously published *PLMVd* variants (Pelchat *et al*., 2000), stem P2 showed less variability. Our data suggests that stem P3 belongs to the left domain, and the right domain is limited to stems P4 to P9. Moreover, we found only two co-variations in P3 stem and one co-variation in P11 stem in the Iranian *PLMVd* variants, and this contrasts with previous analyses that failed to detect co-variation in the P3 stem (Fekih Hassan *et al*., 2007) and is in agreement with previous analyses that failed to detect co-variation in the P6 and P7 stems (Pelchat *et al*., 2000), and in the P4 stem (Fekih Hassan *et al*., 2007). The co-variations for the formation of a pseudoknot by the nucleotides of the positions 339 to 2 in the loop 11 with those of the positions 64 to 67 in the L1 and L11 loops (Figure 4) has been observed previously in Spanish isolates (Ambros *et al*., 1998, 1999; Fekih Hassan *et al*., 2007). Accordingly, it has been argued that the Iranian isolates might have originated from the Spanish isolates. Further support for this conclusion comes from placement of the Spanish isolate gds 15-10 as with the Iranian isolates in the same clade. The results of the co-variations for the formation of this pseudoknot agreed with those of the phylogenetic analysis (Figures 2 and 4).

In conclusion, this study was conducted for the first time in Iran to assess the incidence of *PLMVd* in apricot, sweet cherry, sour cherry and nectarine hosts and this is the first report of infection by *PLMVd* in these hosts. Alignment and phylogenetic analysis of the peach and non-peach

sequences in this study showed that they were clustered in almost all phylogenetic groups, indicating that *PLMVd* variants are not host- or geographic origin-specific. We also showed that the Iranian variants belong to Group II, which shows symptoms in their hosts. Therefore, to limit further dissemination of graft-transmissible diseases in the country, implementation of a national certification program is highly desirable.

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مشخصات مولکولی واریانت‌های ویروئید موزاییک نهان هلو جدا شده از باغات هسته‌دار استان کردستان، ایران

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چکیده

به منظور بررسی حضور و تعیین مشخصات مولکولی *PLMVd* در غرب ایران (استان کردستان)، طی بهار و تابستان ۱۳۹۴ و ۱۳۹۶ تعداد ۱۳۲ نمونه برگی از هلو، آلو، زردآلو، شلیل، گیلاس، بادام و آلبالو از باغات استان جمع‌آوری شد. استخراج اسید نوکلئیک کل با استفاده از روش سیلیکا و سنتز cDNA با استفاده از آغازگرهای تصادفی شش‌تایی انجام شد. PCR با استفاده از آغازگرهای اختصاصی این ویروئید منجر به تکثیر قطعه مورد انتظار حدود ۳۵۰ جفت باز در ۳۶ نمونه شد. به منظور بررسی صحت قطعات تکثیر یافته و تعیین مشخصات مولکولی آن‌ها، فرآورده ۱۷ نمونه/درخت پس از اتصال به پلاسمید pTG19-T و همسانه‌سازی در باکتری *E. coli* DH5α تعیین توالی شدند. توالی‌های به دست آمده *PLMVd* در بانک ژن و هم‌ردیف سازی این توالی‌ها با سایر توالی‌های این ویروئید نشان داد که ۹۴/۳–۱۰۰٪ تشابه بین جدایه‌های این تحقیق و ۷۹/۲–۱۰۰٪ با سایر جدایه‌های گزارش شده شباهت نوکلئوتیدی وجود دارد. در بررسی تبارزایی، جدایه‌های حاصل از این تحقیق با ۳۲ جدایه منتشر شده قبلی در دو گروه تبارزایی قرار گرفتند که جدایه‌های این تحقیق در گروه دوم (جدایه‌های ایجاد کننده موزائیک) با جدایه‌های از هند، چین، اسپانیا، تونس، استرالیا و ایران هم گروه شدند. همچنین ارتباطی بین منشا جدایه‌ها و میزان شباهت نوکلئوتیدی آن‌ها به دست نیامد. ساختار ثانویه ترسیم شده برای برخی جدایه‌های این تحقیق حاکی از ساختارهای جدید در این جدایه‌ها بود. بنابر اطلاعات موجود

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این اولین گزارش از ردیابی *PLMVd* از میزبان‌های هلو، شلیل، گیلاس، آلو، زردآلو، بادام و آلبالو از استان کردستان و از میزبان‌های زردآلو، گیلاس، بادام و آلبالو از ایران می‌باشد.

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