






RESEARCH ARTICLE

First Detection and Molecular Characterization of *Peach latent mosaic viroid* in a New Natural Host: Walnut (*Juglans regia* L.)

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ABSTRACT

Peach latent mosaic viroid (PLMVd) occurs naturally in peach and nectarines worldwide including North and South America, Europe, Asia and Australia with high infection frequencies. Here, our experimental findings revealed for the first time PLMVd infections in walnut (*Juglans regia* L.) in the world. Twelve walnut leaves, all from symptomless plants, were obtained from a nearby walnut germplasm collection in Malatya (Turkey). Genomic RNAs were extracted using a commercial RNA isolation kit and the complete viroid genome was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) assay using end to end primer pair. The PCR amplicons of expected size (approx. 0.34-kb DNA fragments) were cloned into pGEM-T Easy vector and transformed into competent *Escherichia coli* strain JM109. Purified plasmids were sequenced bidirectionally. Of the 12 walnut specimens tested by RT-PCR, 4 were found infected by PLMVd detected in mid-summer of 2018 and in autumn of 2019. Analysis of nucleotide sequence of two walnut isolates (Acces. no: MN857143-MN857144) confirmed the presence of PLMVd and shared 96.6-98.8% similarity (Acces. no: MN857143) and 97-98.8% similarity (Acces. no: MN857144) with peach, nectarine and cherry isolates of the world. By identifying walnut as an alternate host, we confirm that PLMVd potentially may infect other fruit trees cultivated worldwide. Here, we also report molecular features of these two distinct PLMVd-walnut isolates detected in Malatya (Turkey).

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Introduction

Viroids are the smallest and simplest nucleic acid-based pathogens in certain cultivated and non-cultivated wild plant hosts. They are circular, single-stranded RNAs and a unique class of infectious agents causing significant losses in agriculture (Flores et al., 2000; Diener, 2003; Flores et al., 2005). The covalently closed circular noncoding RNAs of viroids replicate in the nucleus or chloroplast and have genome sizes of 250-400 nt. The replicating individuals then traffic from cell

to cell to establish systemic viroid infection in the infected host tissues and organs (Ding, 2010).

Peach latent mosaic viroid (PLMVd) belongs to the genus *Pelamoviroid* in the family *Avsunviroidae*. All the members in this family replicate in the chloroplast, have a conserved hammerhead structures and adopt a branched or quasi rod-like secondary structure. It is a group A viroid which has a self-cleaving hammerhead motifs necessary for its reproduction (Hernandez and Flores, 1992; Ding, 2010).

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PLMVd has been considered as the causal agent of peach latent mosaic (PLM) disease. It was first detected in France (Desvignes, 1976), which were characterized by chlorosis and peach blotch. Later, the agent has been reported in other stone fruit growing countries including US, Italy, Spain, Austria, Turkey, Greece, Yugoslavia, Romania, Morocco, Algeria, China, Japan, Chile, and in Australia (Desvignes, 1986; Flores and Llácer, 1988; Flores et al., 1990; Albanese et al., 1992; Flores et al., 1992; Fiore et al., 2003; Torres et al., 2004; Shamloul et al., 1995; Di Serio and Ragozzino, 1995; Skrzeczkowski et al., 1996; Di Serio et al., 1999; Pelchat et al., 2000; Sipahioğlu et al., 2006; Gumus et al., 2007; Gazel et al., 2008). Although it remains latent for many years following the first infection, PLMVd causes mosaic, bud necrosis, vein banding, blotch and death of branches in peach trees (Desvignes, 1999).

PLMVd is known to have a narrow host range. The pathogen naturally infects peach (*P. persica*) and peach hybrids, Japanese plum (*Prunus salicina* Lindl.), apricot (*Prunus armeniaca*), sweet cherry (*P. avium*), European plum (*P. domestica* L.) and cultivated and wild pears (*Pyrus communis* and *P. amygdaliformis*, respectively), which are all in the family *Rosaceae* (Shamloul et al., 1995; Faggioli et al., 1997; Hadidi et al., 1997; Giunchedi et al., 1998; Osaki et al., 1999; Kyriakopoulou et al., 2001; Giunchedi et al., 2011). PLMVd is readily transmissible by grafting, mechanically transmitted with pruning tools contaminated with the viroid by cutting infected plants and experimentally by *Myzus persicae*, but not through seed (Desvignes, 1986; Flores et al., 1990; Hadidi et al., 1997).

There are few studies dealing with the natural and experimental host ranges for PLMVd (Flores et al., 2000). For many years, PLMVd was believed to be restricted to the peaches and nectarines (Desvignes, 1976; Desvignes, 1999), although it had been reported to infect other stone fruits including almond, apricot and cherry plants (Shamloul et al., 1995; Hadidi et al., 1997; Faggioli et al., 1997; Giunchedi et al., 1998; Osaki et al., 1999; Kyriakopoulou et al., 2001; Giunchedi et al., 2011). The present study reveals the natural occurrence of PLMVd, a viroid not previously reported in walnut, and describes molecular characteristics of two PLMVd-walnut isolates based on full genome sequence analysis.

Materials and Methods

Collecting Plant Material

In June and July of 2018, twelve non-symptomatic fresh and young walnut (*Juglans regia* L.) leaf samples were randomly collected from walnut orchard in Malatya (Turkey) and used in RT-PCR assay and molecular cloning. In September of 2019, the leaves of healthy and PLMVd-infected walnuts were analyzed similarly, identified in previous tests. RNA from a healthy walnut tree was used as negative control. An isolate of PLMVd was used as a positive source for diagnosis of the agent identified from preliminary tests of plants from the same orchard in 2018.

Nucleic Acid Extraction, cDNA Synthesis and RT-PCR Assay

Total RNA was isolated using a commercial plant RNA extraction kit (GeneJet™ Plant RNA Purification Kit, Thermo Scientific, Waltham, MA USA) following the manufacturer's instructions and used to synthesize the complementary DNA (cDNA) from symptomless walnut leaf tissues using reverse primer. A total of 2 µl of purified RNA was used to synthesize the first-strand cDNA and *Moloney murine leukemia virus* (MML-V) reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's instructions. Genome specific PLMVd-F-5'-AACTGCAGTGCTCCGAATAGGGCAC-3' PLMVd-R-5'-CCCAGATAGAAAGGCTAAGCACCTCG-3' primers (Loreti et al., 1999) were used for amplification, detection and molecular characterization of PLMVd isolates in samples prepared from walnut leaf tissues by reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR assay was performed in a 50 µl reaction volume in a sterile microfuge tube containing 10 µl of 5X GoTaq Green Buffer (Promega, Madison, WI, USA), 1 µl of dNTP (20mM each), 1 µl of each forward and reverse primer (100 mM each), 3 µl of MgCl₂, 2 µl of cDNA template, 0.4 µl of Taq DNA polymerase enzyme (Promega, Madison, WI, USA) and 31.6 µl nuclease-free water. PCR was performed in a Thermo Scientific Arktik Thermal Cycler (Waltham, MA, USA) and the cycling parameters were as follows: initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 2 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. In diagnosis of PLMVd isolates by RT-PCR assay, RNA preparations from walnut trees identified negatively and positively from preliminary tests, were used as positive and negative controls. Fifteen µl of RT-PCR product of each amplification was electrophoresed on 2% agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and stained with Pronasafe nucleic acid staining solution (CondaLab).

Molecular Cloning of PLMVd Sequences of Walnut Isolates

The RT-PCR products from PLMVd infected walnut trees obtained by using Phusion Taq polymerase and subsequent Adenine tailing were ligated onto pGEM-T Easy cloning vector (Promega, Carlsbad, CA), following the manufacturer's instructions. Recombinant plasmids were transformed to *Escherichia coli* JM109 by electroporation using micropulser. Before plasmid DNA purification from bacterial cultures using miniprep kit (Fermentas, Vilnius, Lithuania), Colony-PCR was implemented to confirm the presence of inserts in recombinant plasmid bearing bacterial colonies. Purified recombinant plasmids were then sequenced bi-directionally.

Phylogenetic Analyses and Prediction of the Most Stable Secondary Structure of the Identified PLMVd Variants

The assigned sequences were compared with the sequences of viroid isolates retrieved from NCBI GenBank. For the sequence similarity and phylogenetic analysis multiple alignments of related sequences were created using CLC main workbench program (Denmark). Phylogenetic relationships

among PLMVd sequence variants were assessed using neighbor-joining method using CLC Main Workbench Software (CLC bio, Denmark) and phylogenetic trees were constructed with 1000 bootstrap analysis to estimate support for inferred clades. For the calculation of the most stable secondary structure of the PLMVd isolates, computer analysis was performed using the mfold structure prediction package of CLC Workbench program (CLC bio, Denmark).

Results and Discussion

Detection and Identification of PLMVd Isolates

PLMVd infections in symptomless walnut trees in Malatya province (Turkey) were detected by RT-PCR using genome specific primers in 4 out of 12 leaf samples (Figure 1). PCR amplicons of the expected size (approximately 0.34 kb) were observed in agarose gel specific to PLMVd from walnut samples. No PCR amplicon was visualized in the negative control when RNA from healthy walnut plants was used as template (Figure 1). Nucleotide comparisons revealed that

both isolates, determined by RT-PCR, are PLMVd. RNA preparations from fresh walnut leaves were used to synthesize full length PLMVd complementary DNAs, then were cloned and sequenced. The presence of the PLMVd clones on plasmids were confirmed by Colony-PCR (Figure 2). Nucleotide sequence comparisons of PLMVd isolates (PLMVd-M1 and M2; sequences deposited in GenBank with the accession numbers of MN857143 and MN857144, respectively) from Malatya province showed a low level of variability with the world isolates ranging from 96.6% to 98.8%. The full genome sequence analyses of all Turkish PLMVd-walnut isolates revealed a close relationships with each other sharing a strong homology. Only two nucleotide differences were detected between the two walnut isolates. PLMVd-walnut isolates clustered with the isolates of Turkish origin and with those isolates from Far East countries such as China and South Korea (Figure 3). The walnut isolates were clustered into a separate clade consisting Tunisian (Acces. no: HM185109), Chinese (Acces. no: 898808) and South Korean (Acces. no: JN680784) origin (Figure 3).

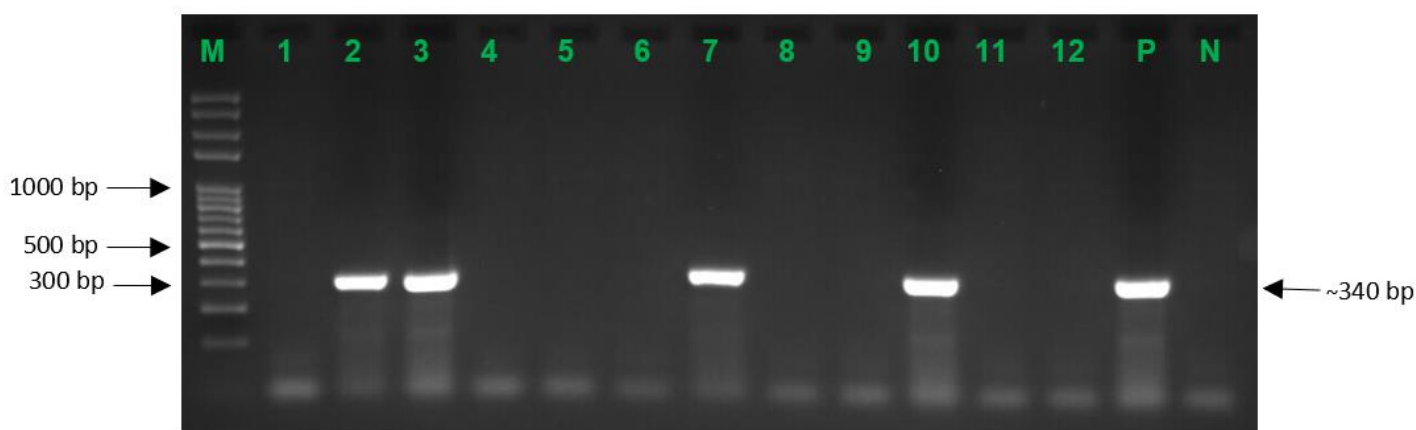


Figure 1. Detection of PLMVd by RT-PCR using genome specific primer pairs. Lanes 1-12 represent the tested walnut samples, lane 2, lane 3, lane 7 and lane 10 (0.34 kb) are positively reacted samples; M: 3.000 bp molecular markers, P: positive control, N: negative control

With this work, for the first time, the walnut isolates of PLMVd were sequenced. The nucleotide sequences of the PLMVd-walnut isolates were different. One isolate was exhibited nucleotide mutation on its genome, although the 2 sequenced variants had a uniform genome size of 339 nt. This difference did not alter the formation of branched secondary structure and hammerhead arm of both isolates when predicting the most stable secondary structure. The substitution was located at positions 243 of monomeric unit of PLMVd-Walnut M2 isolate.

The resulting phylogenetic tree showed that the walnut isolates clustered separately and exhibited similarity with some world isolates including Turkish PLMVd peach isolates. This similarity proved when the walnut isolates were compared with 13 previously characterized PLMVd world isolates (Table 1) (Figure 3).

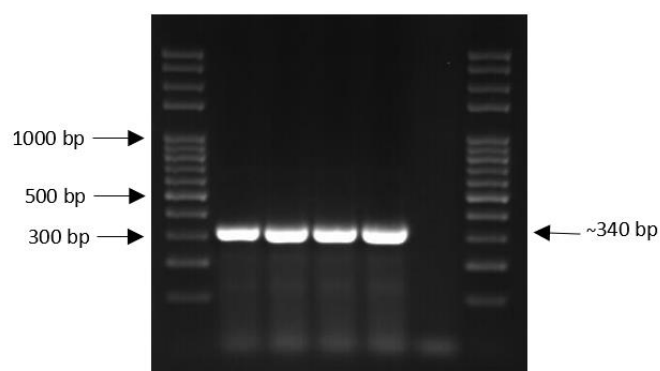


Figure 2. Colony PCR confirmed the presence of the 4 isolates of PLMVd in each purified recombinant pGEM T Easy vector

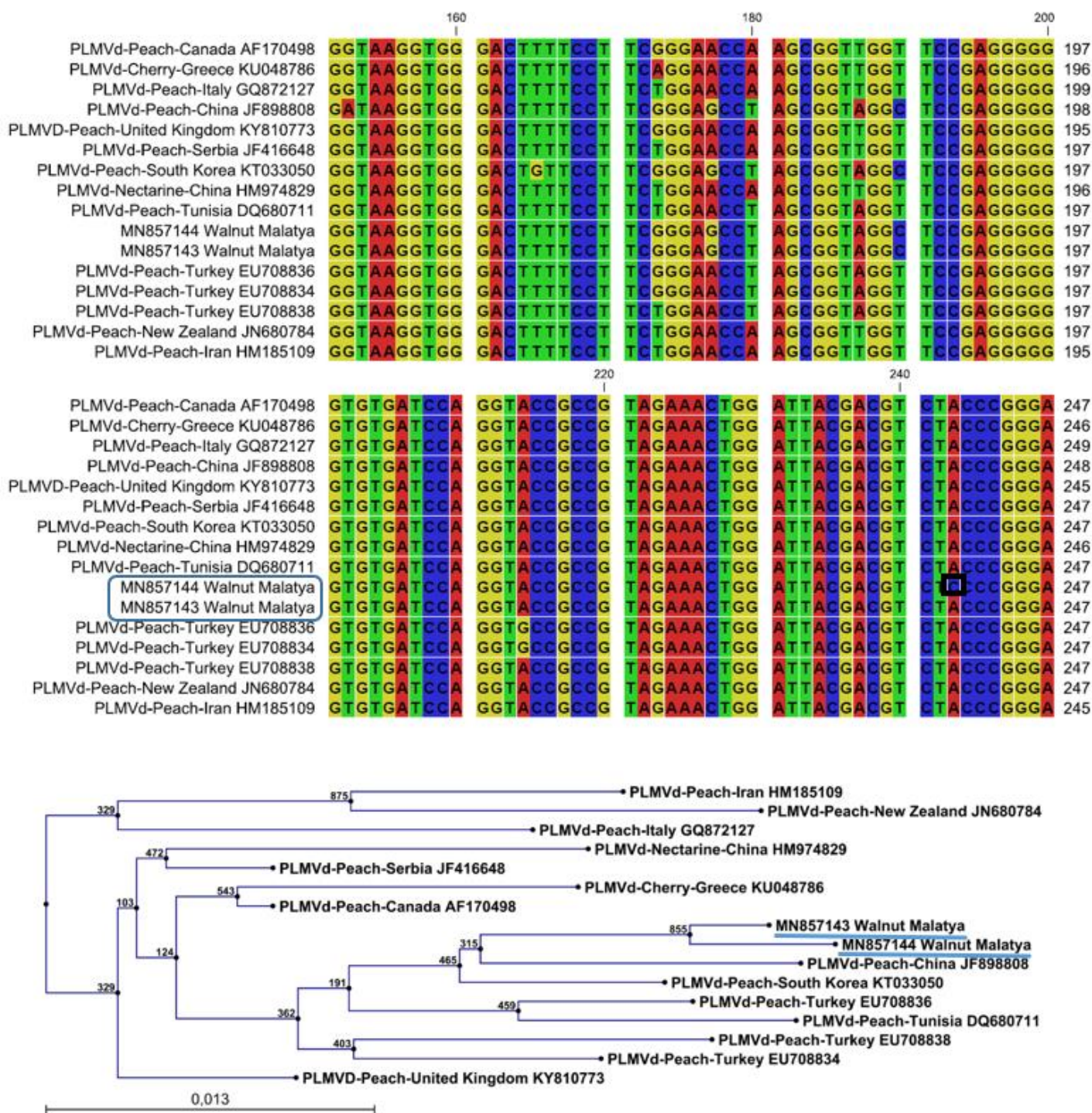


Figure 3. Multiple sequence alignment and the phylogenetic tree of the full-length of PLMVd walnut isolates and the sequences of isolates retrieved from databases and the reference PLMVd isolate (M83545) (Hernandez and Flores, 1992). A nucleotide mutation with respect to the world isolates is seen on the sequence in box

The most stable secondary structures were predicted for both PLMVd variants using the mfold structure prediction function of CLC Main Workbench Software. Calculating free energies of all possible secondary structures and retaining one of lowest energy (i.e., the most stable) is the basis of predicting thermodynamically the most stable molecule (Chastain and Tinoco, 1991). The branched topology of two PLMVd isolates were similar in these analyses because they were identical at all potentially molecular informative sites.

For better description of secondary structure, the stems were numbered starting from first stem-loop structure (hairpin) and from first nucleotide (P1) of PLMVd monomeric strand produced from concatemers via ribozymes. The secondary structure of both isolates were similar to that of reference PLMVd isolate (M83545) (Hernandez and Flores, 1992) (Figure 4). Secondary structure of walnut isolates comprise a total of 7 stem-loop structures (hairpins). Based on this model, 28.9%

nucleotides are located in single-stranded region and the remaining 71.1% of nucleotides are in double-stranded region.

On the secondary structure of walnut isolates, 4-bp nucleotides ${}_{63}\text{GCGG}_{66}$ (stem P1) and ${}_{9}\text{CCGC}_{99}$ (stem P2) are perfectly conserved among both PLMVd isolates seem to base-pair to form a pseudoknot as reported previously (Bussière et al., 2000) (Figure 5).

Here, we have addressed the PLMVd natural variants from walnut. The host range of PLMVd is known to be restricted to the *Rosaceae* family (Haddidi et al., 1997). However, these preliminary molecular studies have now been confirmed the natural occurrence of PLMVd in walnuts. The natural host *Juglans regia*, also called English walnut, the Persian walnut, common walnut, Madeira walnut or Carpathian walnut, is a member of the plant genus *Juglans* in the family *Juglandaceae*.

The molecular characteristics of walnut isolates were similar from one another. However, they were slightly differed from PLMVd sequences retrieved from databases. The high degree of similarity, found in PLMVd isolates from walnut, preserved the most conserved features. Both isolates exhibited a hammerhead structure and a branched secondary structure of lowest free energy. Contrary to our findings, Rodio et al. (2006) reported PLMVd isolates from two peach trees (*Prunus persica* (L.) Batsch., cultivars Maycrest and Springcrest), having different sequence characteristics and pathogenic properties, adopting a similar secondary structure. Gazel et al. (2008) reported a high mutational rate in Turkish PLMVd peach isolates. The authors further conducted mFold analysis to determinate if these mutations could influence the secondary structure of detected isolates revealing that the all predicted the most stable secondary structures were highly conserved.

Table 1. Nucleotide length, accession number and list of PLMVd sequences analyzed in this study

Origin of PLMVd isolate	Host	Nucleotide length	Accession number	Reference
PLMVd-Walnut-M1	Walnut	339	MN857143	This study
PLMVd-Walnut-M2	Walnut	339	MN857144	This study
Iran	Peach	337	HM185109	Yazarlou et al. (2012)
New Zealand	Peach	337	JN680784	Guy (2013)
Italy	Peach	339	GQ872127	Luigi et al. (2009)
South Korea	Peach	339	KT033050	Jo et al. (2015)
China	Peach	338	JF898806	Fan et al. (2011) (Unpublished)
Turkey	Peach	339	EU708834	Gazel et al. (2008)
Turkey	Peach	339	EU708836	Gazel et al. (2008)
Tunisia	Peach	339	DQ680711	Fekih Hassen et al. (2007)
Greece	Cherry	338	KU048787	Boubourakas et al. (2009)
Spain	Peach	337	M83545	Hernandez and Flores (1992)
China	Nectarine	337	MH974826	Xu and Lu (2019)
United Kingdom	Peach	337	KY810773	Pecman et al. (2017)

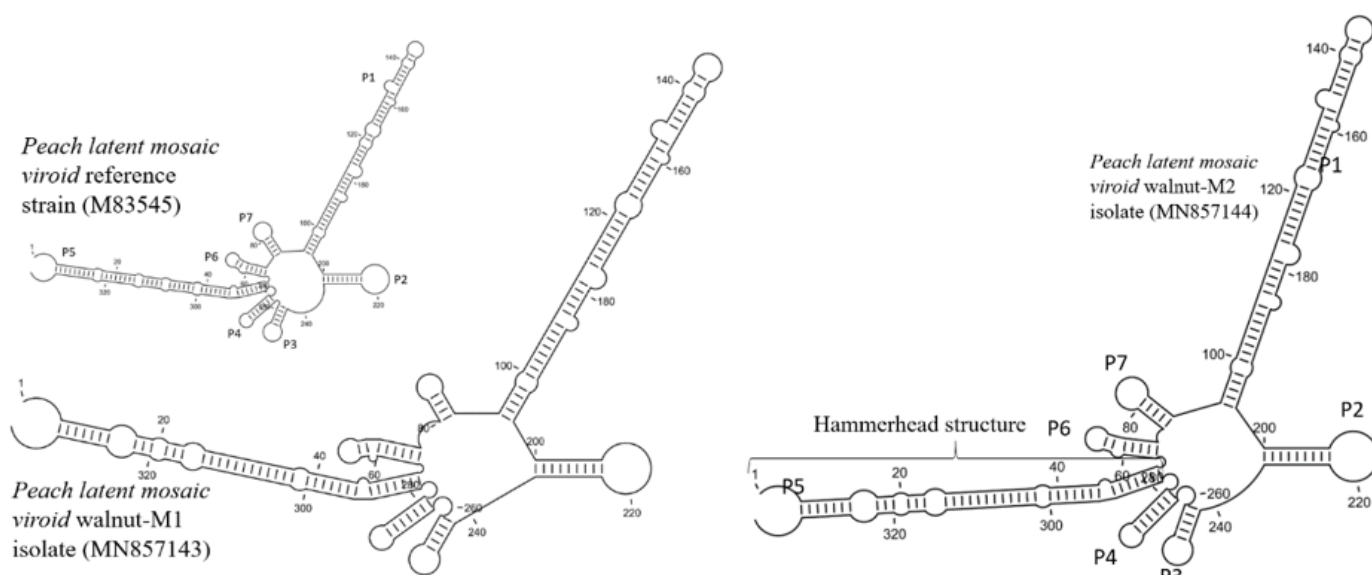


Figure 4. Sequence of Peach latent mosaic viroid (PLMVd) walnut variants and the reference isolate of PLMV folded in the secondary structure of lowest free energy predicted by mfold function of CLC main workbench software

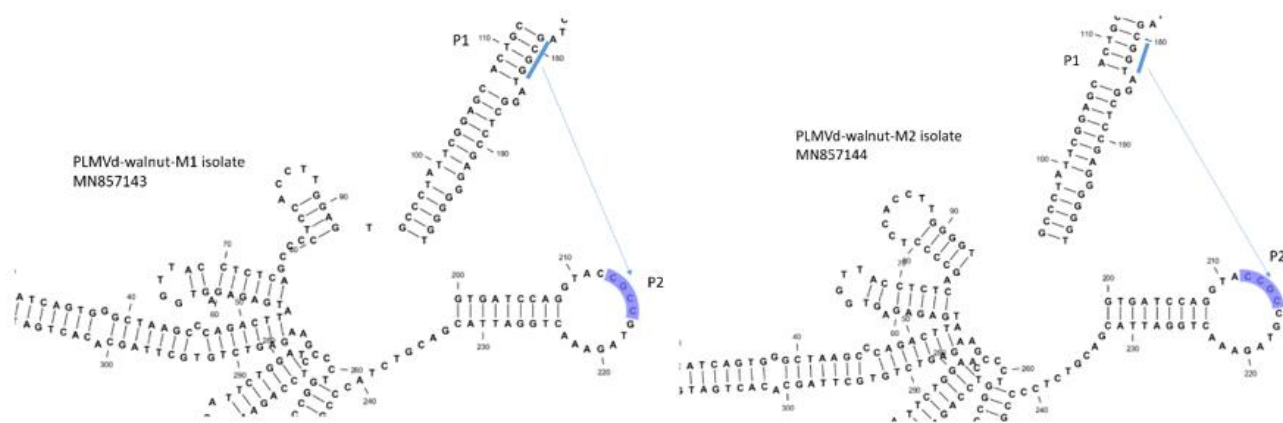


Figure 5. Non interacted conserved 4-bp nucleotides on stem-loop structure (hairpin) P1 and P2 of *Peach latent mosaic viroid* walnut isolates indicated by a line and highlighted nucleotides

No pseudoknot was observed on the secondary structure of the PLMVd-walnut isolates. The conserved nucleotides $_{63}GCGG_{66}$ and $_{96}CCGC_{99}$ are located in double-stranded region of P1 stem-loop structure and at the center of the loop on P2 stem, respectively, giving no possibility to form a pseudoknot. Bussière et al. (2000) reported base composition of this 4-bp sequence is perfectly conserved among all PLMVd variants, and identified the presence of a pseudoknot and named P8 in a mutant PLMVd-peach isolate.

The genetic analyses of two PLMVd walnut isolates resulted in the demonstration that the sequence similarity of the PLMVd-walnut isolates with the world isolates ranged between 96.6% to 98.8%. This range is higher than the accepted arbitrary level of 90% sequence identity (Flores et al., 1998), which the main criterion for the identification of viroid species. Flores et al. (2006) reported a high level of progressive accumulation of point mutations in PLMVd variants. Glouzon et al. (2014) reported the presence of different variants of PLMVd exhibiting up to ~20% of mutations on their genomes.

PLMVd have been transmitted by pruning tools, grafting and by aphids (Flores et al., 2005). Although the pollen transmission of PLMVd has been reported particularly in peaches (Barba et al., 2007), we did not conduct a study to understand the other possible ways of transmission such as pollen, seed and root contact in walnut, which could be responsible from disease spread.

Even PLMVd seems to be latent in walnut, efforts should be made to the eradication of this pathogen in order to avoid the establishment of field reservoirs that, potentially, could be transmitted to susceptible crops such as peach, nectarine, almond and apricot. The contaminated pruning tools may play a role in viroid spread in commercial orchards. Flores et al. (1992) reported a low percentage of experimental transmission of PLMVd by the aphid *Myzus persicae*, however the natural transmission by this vector has never been studied. Because the natural spread of PLMVd in an orchard could be by pollen transmission (Barba et al., 2007) or by grafting, the possible hosts might serve as PLMVd reservoirs for further pollen or

graft transmissions. However, additional studies are needed to determine experimental and natural host (herbaceous and woody) ranges and possible pollen and seed transmission of the PLMVd-walnut isolates where PLMVd isolates were identified in the affected area. Additional studies are required to determine the incidence, distribution, economic impact and insect vectors of PLMVd in walnut orchards. The present study reports on the identification of walnut as a new host of PLMVd and molecular characteristics of walnut isolates based on full genome sequence analysis. To the best of our knowledge, this is the first record of infection of *Juglans regia* by PLMVd.

Conclusion

The information presented in this study clearly shows that PLMVd is the first viroid disease of walnut (*Juglans regia* L.) in the world identified by reverse transcription-polymerase chain reaction (RT-PCR) and complete sequence analyses by molecular cloning. The systemic infection of PLMVd produced no visible symptoms on walnut tree. Further studies are needed to investigate possible transmission ways, geographical distribution and economic relevance of PLMVd in walnut plantations.

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