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RESEARCH ARTICLE

IN SILICO ANALYSIS OF GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS OF PEACH LATENT MOSAIC VIROID (PLMVD) POPULATIONS IN TÜRKIYE, GREECE AND ITALY

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ABSTRACT

Peach latent mosaic viroid (PLMVd) is a chloroplast-replicating RNA that infects peach trees and is known to cause a range of symptoms in its native host, including peach calico and peach mosaic. In this study, 58 isolates from Türkiye and Greece, were subjected to the haplotype network and phylogenetic analysis. A total of 51 haplotypes were detected out of the 58 analyzed sequences, resulting in a haplotypic diversity of 0,99516. Among the populations, the highest number of haplogroups was seen in the Italian population. Phylogenetic analysis revealed two main clusters. Virtual RFLP demonstrated that the BstEII endonucleae can differentiate some of the isolates. These findings demonstrate the variability in the genetic diversity of the populations of Peach latent mosaic viroid in different geographical regions and the importance of identifying and preventing its spread. Therefore, peach farmers in these countries must monitor their crops regularly for the presence of the viroid and take appropriate measures to prevent its spread and manage its impact on their yields.

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INTRODUCTION

A distinct class of infectious agents limited to the kingdom of plants are called viroids. They are made up entirely of a tiny, circular, single-stranded, non-protein-coding RNA that, in some hosts, may replicate independently(Flores *et al.*, 2006). Peach latent mosaic viroid (PLMVd) which belongs to the type species of genus *Pelamoviroid* in the family *Avsunviroidae*is a chloroplast-replicating RNA that infects peach trees and is known to cause a range of symptoms in its native host, including peach calico and peach mosaic(Kyriakopoulou *et al.*, 2017). Understanding the genetic diversity and phylogenetic relationships of PLMVd populations in different regions is crucial for devising effective management strategies for this viroid. The genetic diversity and phylogenetic analysis of PLMVd populations in different regions can be studied using molecular techniques such as quantitative real-time PCR and PCR (Hassan *et al.*, 2009). These techniques have been used to detect and quantify PLMVd in peach trees and can be valuable for analyzing the genetic diversity of PLMVd populations in different geographical regions. Additionally, high-throughput sequencing of small RNAs has been employed to identify viromes of peach trees, which can aid in understanding the diversity and distribution of PLMVd variants (Barath *et al.*, 2022). To gain insights into the genetic diversity and phylogenetic analysis of Peach latent mosaic viroid (PLMVd) populations in silico approaches such as nucleotide diversity, identifying single nucleotide polymorphisms (SNPs), viroid secondary structure prediction can be employed (Avina-Padilla *et al.*, 2015; Sanjuan, 2006). They can provide valuable insights into the evolutionary dynamics and spread of this viroid.

MATERIALS AND METHODS

Viroid sequence sources: Sequences of PLMVd viroid isolates used in this study were retrieved from the National Center of BiotechnologyInformation (NCBI). Turkish isolates: EU708829.1, EU708830.1, EU708831.1, EU708832.1, EU708833.1, EU708834.1, EU708835.1, EU708836.1, EU708837.1, EU708838.1, EU708839.1, EU708840.1, EU708841.1, EU708842.1, EU708843.1, EU708844.1, EU708845.1, EU708846.1, EU708847.1, EU708848.1. Greek isolates: KU048779.1, KU048780.1, KU048781.1, KU048782.1, KU048783.1, KU048784.1, KU048786.1, KU048787.1, KU048788.1, KU048789.1, KU048791.1, KU048792.1, KU048793.1, KU048794.1, KU048795.1, KU048796.1, KU048797.1, KU048798.1. Italian isolates: JN377877.1, JN377878.1, JN377879.1, JN377880.1, JN377881.1, JN377882.1, JN377883.1, JN377884.1, JN377885.1, JN377886.1, JN377887.1, JN377888.1, JN377889.1, JN377890.1, JN377891.1, ON513442.1, ON513443.1, ON513444.1, ON513445.1, ON513446.1.

Polymorphism and phylogenetic analysis of PLMVd isolates: The retrieved sequences were aligned using muscle alignment by Geneious Prime® 2023.2.1 software.To analyze sequence polymorphism variable (polymorphic) sites, singleton variable sites and parsimony informative sites tests were performed among PLMVd populations. Phylogenetic analysis was performed using the Tamura-Nei distance model and neighbour-joiningmethod. Apple chlorotic fruit spot viroid(NC_076621.1) was used as an out-group.

Genetic diversity and haplotype network analysis: Several genetic parameters were also examined using DnaSP version 6.12.03(Rozas *et al*., 2017) to gain a better understanding of the genetic structure, diversity, and evolutionary pattern among the dominant species of PLMVd populations in Türkiye, Italy, and Greece. These parameters included N: Number of sequences, S: Number of segregating sites, h: Number of haplotypes, Hd: Haplotype diversity, K: Average number of nucleotide differences, and Pi: Nucleotide diversity. Using PopArt 1.7(Leigh & Bryant, 2015), a haplotype network was built based on the haplotypes found in the PLMVdpopulations. Finally, the trait segment was utilized as an input file for a geographically-based display after being integrated into the nexus data file produced by DnaSP software.

Virtual Restriction Fragment Length Polymorphism (RFLP) analysis: To obtain RFLP restriction patterns of PLMVd isolates, sequences from Turkish, Greek and Italian populations were analyzed for their restriction sites using Geneious Prime® 2023.2.1. A virtual gel of RFLP patterns was created based on the selected enzyme.

RESULTS

Polymorphism analysis: Muscle alignment was used to align (multiple alignment) the associated PLMVd sequences. In PLMVd populations, 111 variable sites were identified, of which 31 were singleton sites and 80 were parsimoniously informative nucleotide sites. Italy has the greatest number of polymorphic and singleton variable sites among these groups, while the Turkish population has the greatest number of parsimony informative sites (Table 1).

Table 1.Polymorphism analysis of PLMVd isolates

Country	Variable (polymorphic) sites	Singleton variable sites	Parsimony informative sites
Türkiye	77	18	59
Italy	79	44	35
Greece	42	22	20
All	111	31	80

Phylogenetic analysis of PLMVd isolates: A phylogenetic analysis was carried out on the 58 sequence variants isolated from Türkiye, Italy and Greece. Apple chlorotic fruit spot viroid (NC_076621.1) was used as an outgroup. All of the PLMVd isolates were collapsed in 2 main clusters. All of the Turkish, Greek and some Italian isolates are clustered in one group (blue) while most of the Italian isolates are clustered in a distinct group(red) (Figure 1).

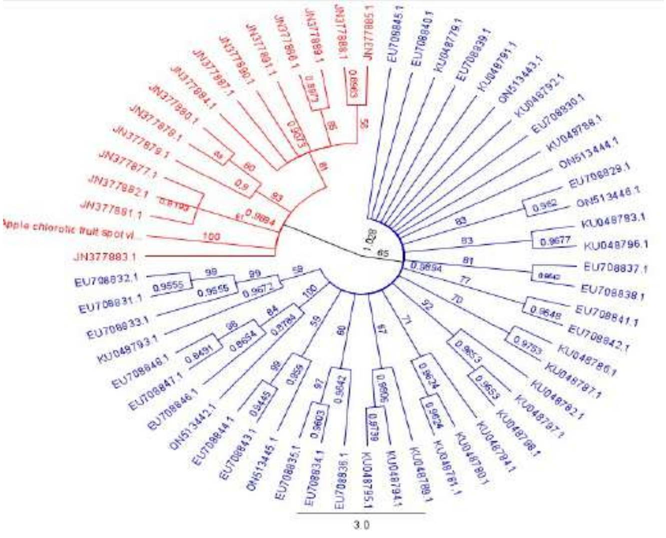

Figure 1. Neighbour-Joining phylogenetic tree of 58 PLMVd isolates from Türkiye, Italy and Greece

Genetic diversity and haplotype network analysis: The sequence variations of PLMVd variants were high with 51 haplotypes detected out of 58 analyzed sequences, resultingin a haplotype diversity of 0,99516. Among the populations, the highest number of haplotypes was seen in the Italian population with the highest haplotype diversity resulting in a 0,99474 Hd value.The average number of nucleotide differences (K) of the populationswas22,51785. The Greek population showed the lowest K value of 9,99346. The Rm value was 11 for both Turkish and Italian populations and 7 for the Greek population (Table 1). Considering the neutrality analysis, Tajima, Fu and Li were found not to be significant (Table 2).Figure 3 shows the haplotype network based on the Median-joining haplotype network of PLMVd haplotypes constructed using PopArt 1.7. The network analysis included 51 haplotypes of PLMVd isolates. The distribution of the haplotypes is depicted in Figure 4.

Table 1. Nucleotide diversity of Peach Latent Mosaic Viroid (PLMVd) isolates from Türkiye, Italy and Greece

Parametre	PLMd Türkiye	PLMVd Italy	PLMVd Greece	All
N	20	20	18	58
S	76	78	42	111
h	16	19	16	51
Hd	0,97368	0,99474	0,98693	0,99516
K	22,53684	18,52632	9,99346	22,51785
Pi	0,06850	0,05631	0,03038	0,06844
Rm	11	11	7	22

N: Number of sequences, S: Number of segregating sites, h: Number of haplotypes,Hd: Haplotype diversity, K: Average number of nucleotide differences, Pi: Nucleotide diversity, Rm:Minimum number of recombination events

Virtual RFLP analysis: For restriction analysis of the sequences, the following endonuclease enzymes were used. AvaII, BstEII, EcoRI, HaeIII, HindIII, KpnI, MboI, MseI, NruI, PstI, RsaI andTaqI. Figure 5shows the restriction sites on the selected sequences. PstI MboI and AvaII were found to have a restriction site on most of the sequences. EU708830.1, EU708843.1, EU708844, EU708847.1, EU708848.1 (Turkish population), KU048792.1, KU048793 (Greek population), ON513442.1, ON513445.1 and ON513446 (Italian population) can be distinguished from other members of the populations by BstEII endonuclease restriction. (Figure 6).

DISCUSSION

Phylogenetic analyses are used to better understand the relationships between different populations of Peach Latent Mosaic Viroid (PLMVd) in various regions. In this study, viroid isolates from Türkiye, Greece and Italy were phylogenetically analysed. The isolates are collapsed into two main groups. Turkish and Greek isolates formed a major group along with some Italian isolates meanwhile the other Italian isolates collapsed into a distinct group. A total of 58 isolates from these countries formed 51 haplotypes. Polymorphic, singleton and parsimony informative sites were detected in this study. It has been hypothesized that the singleton variants are naturally occurring mutations (Hamdi *et al*., 2019). Considering the neutrality test, the values were found not to be significant. The test's justification of Tajima's D test is that, in a panmictic population, the average number of nucleotide changes and the number of segregating sites should not differ, according to the neutral mutation modelThe presence of negative and positive selection is indicated by values that are considerably different from zero, respectively(Gandía *et al*., 2005). Also, the negative D-value provided significant evidence for the viroid sequences' quick growth. As is well known, selection may have been pushing viroid populations into regions of sequence space where neutrality is at its highest during the evolution and diversification of viroids(Hamdi *et al*., 2019).Studies have reported on PLMVd isolates from different peach cultivars and trees, each having unique genetic sequences and exhibiting varying symptoms upon infection(Faggioli & Barba, 2008; Mandic *et al*., 2008). Further research has identified PLMVd isolates in different stone fruit trees in Türkiye, Tunisia and other regions, and determined that the Turkish viroid isolates belong to PLMVd Group III.

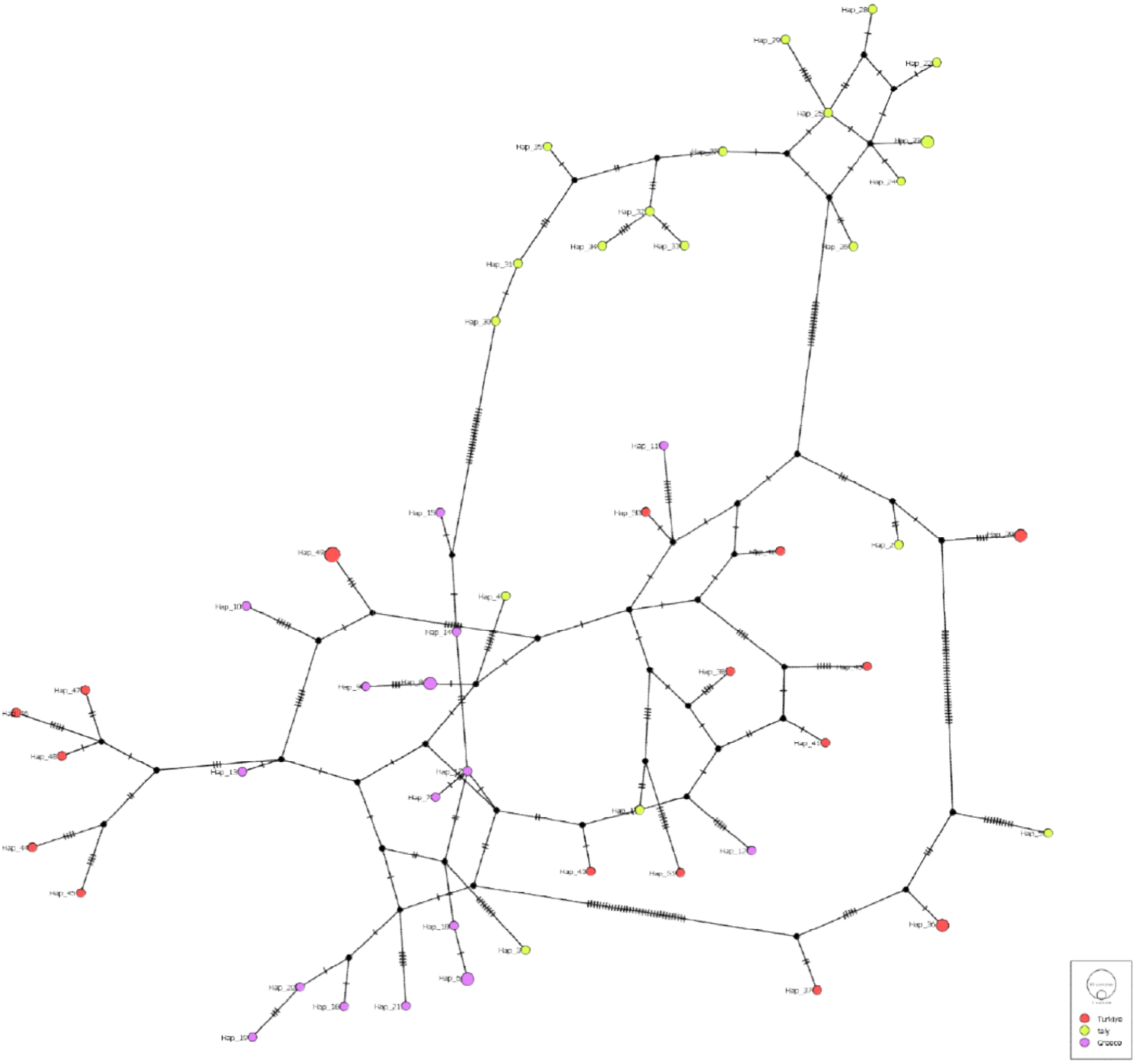

Figure 3. Median-joining haplotype network of PLMVd haplotypes constructed using PopArt 1.7. The network analysis included 51 haplotypes of PLMVd isolates. Each circle represents a unique haplotype, colours (red: Türkiye, yellow: Italy, pink: Greece) represent a country of genome origin, and the size of the circle is proportional to number of genomes included

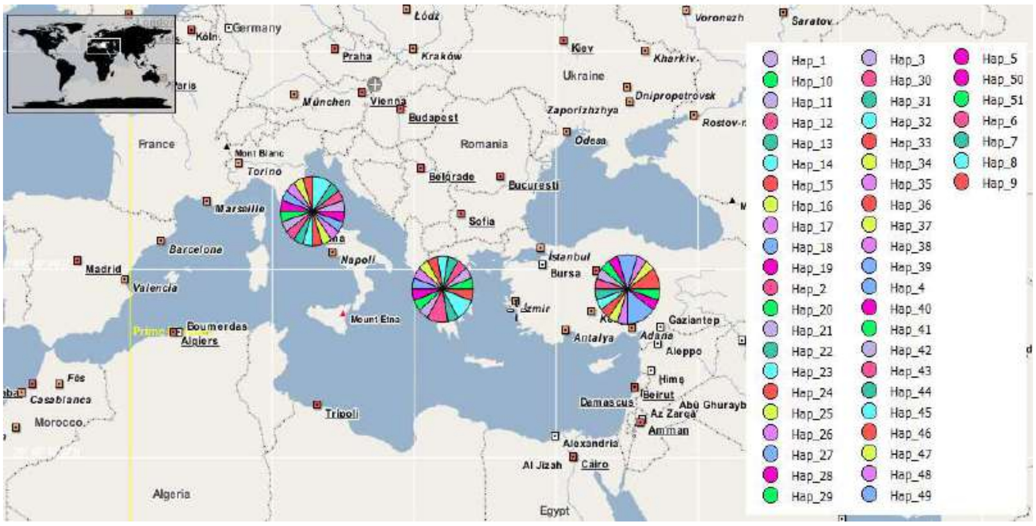

Figure 4. World map showing the distribution of the 51 PLMVd haplotypes represented by a different colour

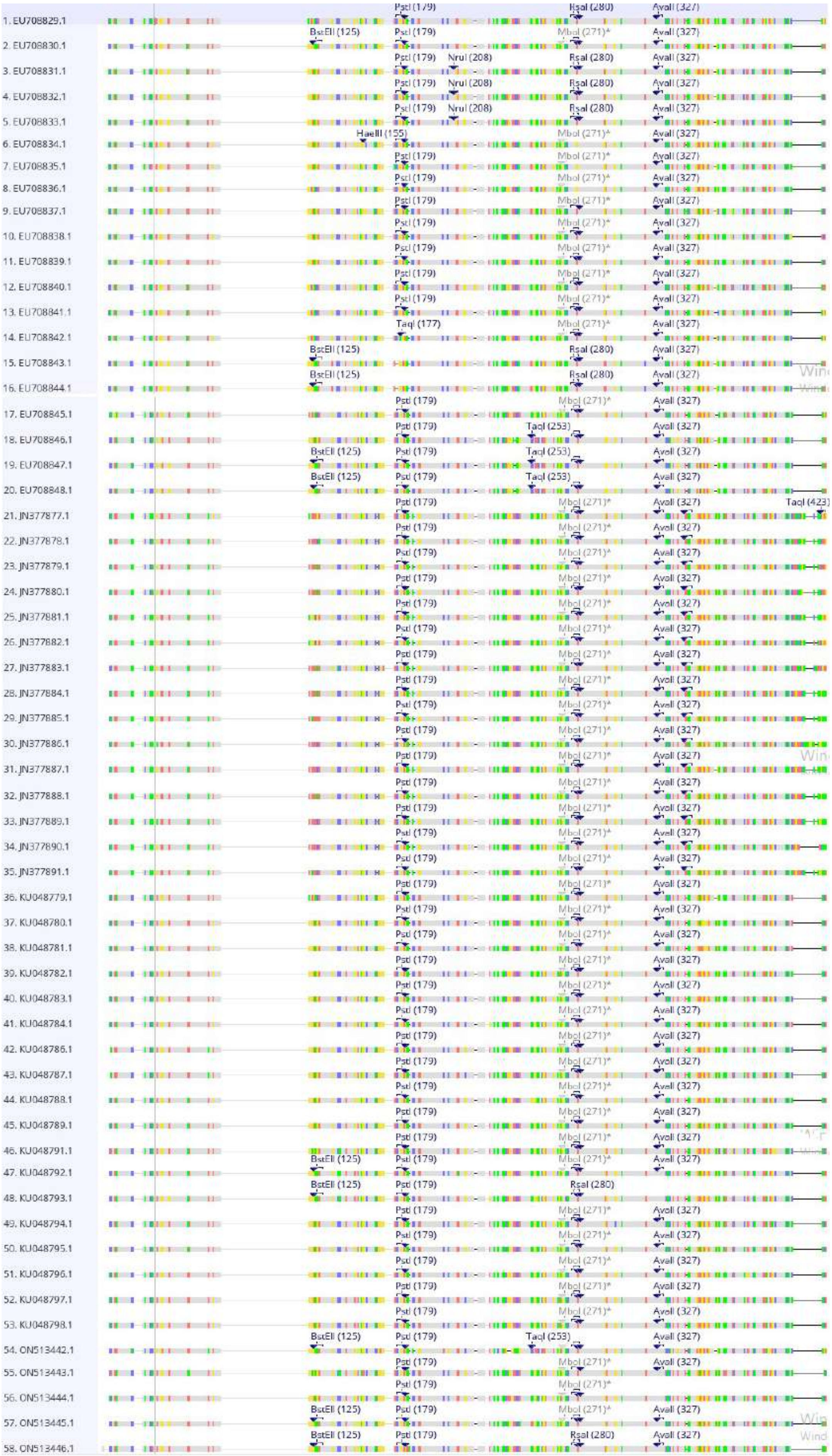

Figure 5. Restriction sites of the selected sequences by following restriction enzymes: AvaII, BstEII, EcoRI, HaeIII, HindIII, KpnI, MboI, MseI, NruI, PstI, RsaI andTaqI

Table 2. Neutrality test for each PLMVd population. NS: Not significant

Neutrality tests	PLMd Türkiye	PLMVd Italy	PLMVd Greece	All	significance
Tajima's D	-0,19979	-1,15530	-1,10377	-0,71862	NS, P > 0.10
Fu and Li's D	0,31307	-1,40971	-1,23269	-0,63249	NS, P > 0.10
Fu and Li's F	0,18477	-1,55538	-1,38734	-0,79749	NS, P > 0.10

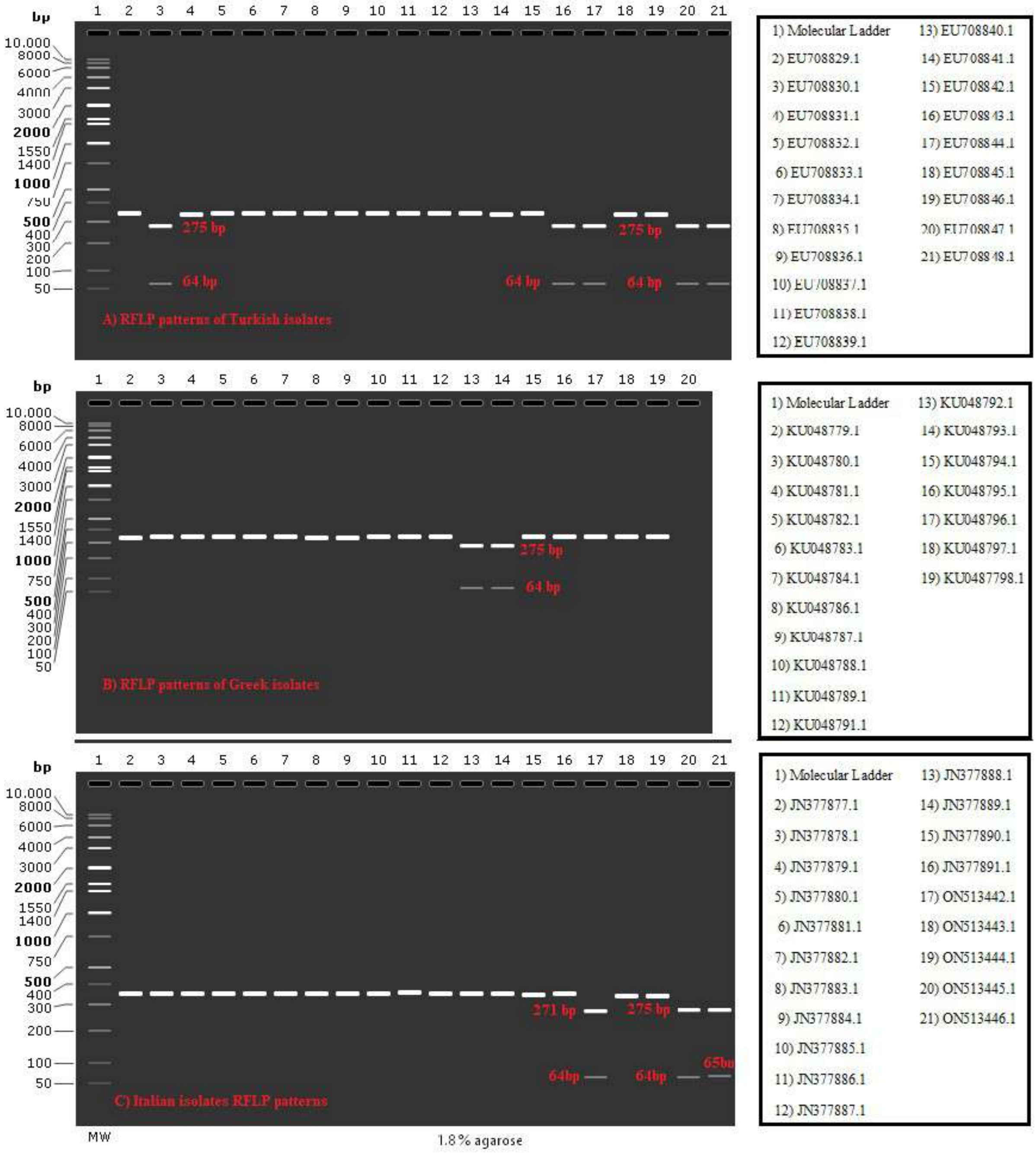

Figure 6.Virtual restriction fragment length polymorphism (RFLP) patterns of*in silico* digestions of isolatesA) Turkish, B) Greek and C) Italian populations using BstEII restriction enzyme

In a study to assess the spread of PLMVd infection, non-isotopic dot-blot hybridization and RT-PCR analysis were used to detect PLMVd in over 400 peach varieties (Rubio-Cabetas *et al*., 2012).Additionally, genetic analyses of PLMVd isolates from different stone fruit trees have shown genetic diversity in the populations(Karagahi & Karagahi, 2020). Furthermore, there have been reports of other viroids, such as apple scar skin viroid (ASSVd), being detected in naturally infected cultivated pear Pyrus communis in Greece, alongside PLMVd (Pedrelli *et al*., 2023). These findings demonstrate the variability in PLMVd populations and how phylogenetic analyses can help reveal the relationships between them.

The genetic diversity of PLMVd has significant implications for peach cultivation in Türkiye, Greece, and Italy. PLMVd shows genetic diversity in these countries, with PPV-Rec isolates having a close phylogenetic relationship with isolates from Italy and Türkiye, suggesting possible cross-border transmission (Pedrelli *et al*., 2023). This may present challenges for peach farmers who need to ensure that their crops remain healthy and productive. In a study conducted in Türkiye, PLMVd infection was found in 15 peach varieties (3%) while HSVd infection was rarely detected only in apricots (0.1%) (Sipahioglu *et al*., 2006).

Similarly, PLMVd and ASSVd were detected in cultivated pears in Greece, indicating the widespread distribution of these viroids in the region(Kaponi *et al*., 2012). Virtual RFLP results demonstrated that some of the isolates from these populations can be distinguished from other members using BstEII endonuclease restriction. The variability among PLMVd isolates further complicates the management of these viroids, as different clones may respond differently to control measures. Therefore, peach farmers in these countries must monitor their crops regularly for the presence of PLMVd and take appropriate measures to prevent its spread and manage its impact on their yields.

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