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Strain identification and sequence variability of plum pox virus in Turkey

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Abstract: Plum pox virus (PPV) is the causal agent of sharka disease of stone fruit trees. Since the late 1960s, PPV infection has been reported in different regions of Turkey. In this study, we aim to discover PPV strains in infected regions and determine its genetic variability in Turkey. For this reason, 612 samples were collected from distant locations, where PPV was previously detected in most cases. First, PPV presence in the samples was tested with serological and molecular methods to confirm the disease. Then 314 positive samples were sequence analyzed at a 664-nucleotide length, including the P3-6K1 gene region, one of the most variable regions among *Potyvirus* species and quite conserved among different strains of a viral species. PPV-D and PPV-T strains were identified mostly in residential gardens, whereas PPV-M was mostly detected in the orchards, except one isolate that was identified as PPV-Rec in Bursa. PPV-T was found to be dominant in the Turkish PPV pool. Estimates of average evolutionary divergence over sequence pairs of P3-6K1 gene regions revealed that the mean intragroup diversity was 0.049 for W; 0.017 for Rec, T, and M; 0.14 for C; 0.012 for Turkish D; 0.009 for global D; and 0.007 for CR.

Key words: Genetic variability, sharka, strain identification, stone fruits

1. Introduction

Plum pox virus (PPV) is considered to be the most devastating disease of *Prunus* spp. (Cambra et al., 2006), and it is one of the 10 most studied plant viruses in molecular plant pathology (Scholthof et al., 2011). PPV is a member of the genus *Potyvirus*, which encompasses approximately 30% of the currently known plant viruses (Gibbs et al., 2008). The virus causes reduced fruit quality and premature fruit drop in susceptible stone fruit crops (peach, apricot, plum, and Japanese plum). After the onset of the viral infection, controlling the virus has been very difficult in stone fruit-producing regions (Cambra et al., 2006).

PPV has a single molecule of a positive, single-sense, RNA genome, approximately 9.7 kb. RNA viruses exhibit rapid evolution and have high mutation rates with large population sizes, resulting in high genetic diversity within the virus populations (Domingo and Holland, 1997). Like other RNA viruses, PPV shows high variability, with nine strains identified to date: Ancestor Marcus (An), Cherry (C), Cherry Russian (CR), Dideron (D), Marcus (M), El Amar (EA), Recombinant (Rec), Turkey (T), and Winona (W). This large number of strains is greater than for any other *Potyvirus* (Garcia et al., 2014). Among the nine strains, PPV-M and PPV-D are considered the two most prevalent groups (Candresse et al., 1998). PPV-M prevalence has been reported throughout southern, eastern, and central Europe (Myrta et al., 2001; Capote et al., 2010). PPV-M identification with reliable sequence analysis was not reported outside of Europe (Garcia et al., 2014). PPV-D is the most prevalent strain in Europe and worldwide and it has been reported in more than 40 countries (Garcia and Cambra, 2007; James et al., 2013). PPV-Rec is a recombination product of the M and D strains, carrying mostly a PPV-D-like genome and known to be present in central and south-central Europe (Cervera et al., 1993; Glasa et al., 2001, 2004; Šubr et al., 2004); in Isparta, Turkey (Candresse et al., 2007); and in Ontario, Canada (Thompson et al., 2009). The other strains were identified in limited regions (Wetzel et al., 1991; Kalashyan et al., 1994; Crescenzi et al., 1997; James et al., 2003; El Maghraby et al., 2007; Ulubaş Serçe et al., 2009; Glasa et al., 2011; Mavrodieva et al., 2013).

Several methods, including biological testing, ELISA, PCR, and sequence analysis, are commonly used for detecting PPV. The development of polyclonal antibody 5B-IVIA and monoclonal antibodies for the four serogroups, M, D, C, and EA strains (Cambra et al., 1994;

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Boscia et al., 1997; Crescenzi et al., 1998; Myrta et al., 1998), made ELISA routine for detecting all PPV strains. PCR has been used to identify the virus universally or strain-specifically by amplifying the variable N-terminal region of the viral coat protein (CP) and the conserved 3′ untranslated region (Wetzel at al., 1991; Levy and Hadidi, 1994; Olmos et al., 1996; Candresse et al., 1998). Sequence analysis is accepted as the most reliable method to identify the virus. Similar to other plant viruses, PPV has been mostly analyzed by sequencing the viral CP gene (Bousalem et al., 1994; Candresse et al. 1998). Typing methods for the P3-6K1 region have also been developed, and the P3-6K1 region has been used for PPV variability studies, allowing PPV subgroup discrimination independent of the CP (Glasa et al., 2002). P1 and P3 are the most variable regions among *Potyvirus* species, whereas P3 is quite conserved among different strains of a given virus (Ward et al., 1992; Aleman-Verdaguer et al., 1997). In a study of four complete PPV sequences conducted by Palkovics et al. (1993), PPV exhibited a higher frequency of amino acid (aa) changes in the N-terminus of CP, P1, and P3, and the C-terminus of HC proteins. The P3-6K1 region of the virus has also been used in several subsequent studies, allowing reliable PPV subgroup discrimination (Glasa et al., 2004, 2005; Matic et al., 2006; Dallot et al., 2011). Šubr and Glasa (2008) reviewed PPV variability and suggested that the P3-6K1 region with a high degree of interstrain heterogeneity may also be used for PPV typing. Recombination is common in plant viruses and should be considered during interpretation of sequence analysis. For example, PPV-Rec is a result of recombination between PPV-M and PPV-D, and its N-terminus comes from PPV-M strains. Therefore, PPV-Rec is genetically closer to the PPV-M strain than to the PPV-D strain based on sequence similarity of N-terminal sequence analysis, whereas it shows closer similarity to D isolates when comparing P3-6K1 regions. Comparing complete sequences is a more reliable approach than partial sequencing (Šubr and Glasa, 2008).

PPV occurrence in Turkey was first detected in 1968, and since then several reports describing PPV incidence have been released. Edirne, a region neighboring Bulgaria, was the first province where PPV was observed in Turkey. Subsequently, it was detected in the Central Anatolia, Marmara, Mediterranean, Aegean, and Black Sea regions (Şahtiyancı, 1969; Kurçman, 1973; Yürektürk, 1984; Erdiler, 1988; Elibüyük and Erdiller, 1991; Elibüyük, 2003, 2004, 2006; Sertkaya et al., 2003; Koç and Baloğlu, 2006; Candresse et al., 2007; Gümüş et al., 2007; İlbağı et al., 2008; Ulubaş Serçe et al., 2009; Akbaş et al., 2011; Ceylan et al., 2014; İlbağ and Çıtır, 2014, Deligöz et al., 2015). ELISA and PCR approaches were mostly performed to detect PPV in Turkey. For example, Sertkaya et al. (2003) performed double antibody sandwich (DAS)-ELISA, reverse transcription (RT)-PCR, and RFLP RT-PCR to characterize Turkish PPV isolates. Double antibody sandwich indirect (DASI)-ELISA and molecular typing showed that 2 of 52 stone fruit samples collected from apricots in Ankara Province were infected with the M strain of PPV. Based on DAS-ELISA typing, Elibüyük (2003, 2004, 2006) reported that many apricots, plums, and peaches are infected with PPV-D, M, and a mix of D and M in Ankara, which is accepted as the most infected region in Turkey. Sequence analysis was also performed for characterization and strain identification of Turkish isolates. First, Glasa and Candresse (2005) analyzed the sequences of a Turkish isolate, and later Ulubaş Serçe et al. (2009) studied a group of Turkish isolates in detail by sequencing full genomes and fragments of the isolates. The incidence of PPV-Rec in Isparta, Turkey (Candresse et al., 2007), and PPV-T infection in almonds in Tekirdağ were also determined by sequence analysis (İlbağ and Çıtır, 2014).

Horticultural plants are very important for the human diet as sources of vitamins, minerals, and dietary fiber and they moreover become a significant part of human life due to their medicinal and environmental uses as well as aesthetics and economic values. The stem, leaf, flowers, roots, and fruits of vegetables and fruit crops have the highest potential of export (Bajpai et al., 2014; Kaczmarska et al., 2015; Mlcek et al., 2015; Wojnicka-Poltorak et al., 2015). Turkey is a horticultural country with high production and consumption of fruits and vegetables. Among fruits, stone fruits have a significant place in Turkey, accounting for 14% of fruit production, the third most significant after pome and citrus fruits (http://faostat.fao.org/). Turkey is the leading apricot-producing country in the world, providing 70% of the dried apricots consumed worldwide, and exports approximately $300 million to $350 million's worth of dried apricots annually. Turkey is also a top producer of cherries and a good producer of peaches, plums, and almonds. Unfortunately, studies show that stone fruits are mostly susceptible to PPV. To date, resistance has been found only in the North American apricot germplasm (Kegler et al., 1998; Martinez-Gomez and Dicenta, 2000; Martinez-Gomez et al., 2000), in Stark Early Orange, Goldrich, Harlayne, Stella, and Harcot. These cultivars are mostly used as resistance sources for apricot breeding. A recent study showed that Turkish apricots mostly lack the North American-type resistance (Gürcan et al., 2015). Therefore, comprehensive knowledge of the prevalence and genetic variability of PPV is important for effective disease management, which is important for the stone fruit industry. Although PPV variability in European countries has been well studied, PPV strain identification with sequence analysis and variability in Turkey has been poorly investigated. PPV strains and variability in Turkey

should be examined and elucidated in more detailed. In the present study, we aim to discover the strains in infected regions and the genetic variability of the virus in Turkey.

2. Materials and Methods

2.1. Sample collection

The following provinces were visited: Ankara, Aksaray, Antalya, Aydın, Balıkesir, Burdur, Bursa, Çanakkale, Denizli, Eskişehir, Isparta, İstanbul, İzmir, Kayseri, Kırşehir, Kırklareli, Konya, Malatya, Manisa, Nevşehir, Niğde, Sivas, Tekirdağ, Yalova, and Yozgat. In total, 612 samples were collected in either early summer or early spring of 2012 and 2013. Five to 10 leaves with symptoms were taken from each tree. Mostly symptom-showing apricot and plum trees were collected, but not all samples with symptoms were collected. Because we did not observe clear symptoms on peach leaves, samples were taken from peach trees only when they were located close to symptom-showing apricot and plum trees. In commercial apricot, peach, and plum orchards, samples were collected from only those orchards that were previously known to have PPV symptoms based on information provided by local agricultural officers. Of the total samples, 469 were apricot, 71 were plum, and 60 were peach. The provinces from which the samples were collected are listed in Table 1. More samples from different neighborhoods of Ankara were gathered because previous papers have reported that Ankara is highly infected.

2.2. Double antibody sandwich indirect-ELISA

Leaves showing PPV symptoms were taken to the laboratory, and a plant extract was prepared according to the procedure of the European and Mediterranean Plant

Table 1. List of sample origins and PPV infection rates.

Location	Sample tree	Number of collected samples	Number of symptom-showing samples	Number of infected samples	Infection rate (%)
Ortaköy/Aksaray	Apricot	73	73	70	96
	Plum	15	15	12	80
	Peach	1	0	0	0
Ankara	Apricot	144	144	140	97
	Plum	14	14	13	93
	Peach	2	0	1	50
Aydın	Apricot	18	18	15	83
Bursa	Plum	15	15	5	33
	Peach	9	4	5	56
Çanakkale	Peach	14	5	12	86
Denizli	Apricot	19	19	15	79
Eskişehir	Apricot	8	4	4	50
Isparta	Peach	12	5	8	67
İstanbul	Apricot	25	25	20	80
	Plum	9	9	6	67
	Peach	10	0	5	50
İzmir	Apricot	10	5	3	30
Kayseri	Apricot	90	90	88	98
	Plum	19	19	15	79
Konya	Apricot	59	59	57	97
	Plum	15	15	11	73
Kırklareli	Apricot	10	1	1	10
Tekirdağ	Apricot	15	15	12	80
	Plum	6	6	6	100
Total		612	560	524	86

Protection Organization (EPPO, 2004). DASI-ELISA was performed using PPV-specific monoclonal antibody 5B-IVIA, PPV-D-specific antibody 4D, and PPV-M-specific antibody AL. DASI-ELISA kits were purchased from Real (Valencia, Spain), and the tests were performed according to the manufacturer's instructions and EPPO protocol (2004). The DASI-ELISA test was performed for 212 samples collected in 2012. The optical densities (ODs) were measured 60 min after adding substrate, at a wavelength of 405 nm, using a PowerWave 200 scanning microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). Three negative samples were used for each 96-well plate. Samples that showed OD values two times higher than the average negative control OD were accepted as positive.

2.3. Reverse transcription-PCR with plum pox virus-specific primers

Total RNA was extracted using a commercial RNA isolation kit (Roche, Indianapolis, IN, USA). Plant extracts prepared for the ELISA test were spun briefly, and 300 µL of the supernatant was used for RNA extraction. RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA. USA). cDNA was constructed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol, with modifications. Briefly, 2 µL of mixture A (100 ng of random hexamers and 1 µL of 10 mM dNTP) was added to 10 µL of RNA (average 30 ng/µL RNA), and the total mixture was heated to 65 °C for 5 min and quickly chilled on ice. Mixture B (4 µL of 5X First-Strand Buffer, 2 µL of 0.1 M DTT, and 1 µL (200 units) of M-MLV) was added to the reaction and kept at 25 °C for 10 min to anneal the primers, 37 °C for 50 min to synthase cDNA, and finally at 70 °C for 15 min to inactivate the reaction.

Initially, PCRs were conducted using primers P1 and P2, which are specific to all strains (Wetzel et al., 1991). Molecular typing for strain identification is done only on the CP gene, using P1/PD and P1/PM. Primers P1/PD and P1/PM were used to identify the PPV-D and PPV-M strains, respectively (Olmos et al., 1997). These primers were developed from the CP gene of the virus. PCR was performed using 2 µL of cDNA, 1.5 µL of 10X Taq polymerase buffer, 12 pM each of forward and reverse primers, 35 µM $MgCl_2$, 27 µM each of dNTPs, 0.2 U of Taq DNA polymerase (Thermo Fisher Scientific Inc.), and water to a total volume of 15 µL. PCR amplification was performed on a T100 Termal Cycler (Bio-Rad Laboratories, Hercules CA, USA) using a PCR program as follows: initial 2 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, with a final extension for 7 min at 72 °C. The products were run on 2% agarose gels prepared with TBE buffer. P1/P2, P1/PM, and P1/PD produce 243-, 198-, and 198-bp amplicons, respectively.

2.4. Sequencing PCR

For the sequence analysis, the genomic region corresponded to nucleotides (nt) 2915–3750 (numbered according to GenBank AJ243957), covering the 3′ terminal part of P3, the entire 6K1 gene, and the 5′ terminal part of the CI gene, was amplified by the PP3/PCI primer pair developed by Glasa et al. (2002a). Fifteen-microliter PCR was performed as described previously. The PCR conditions were the same as in Section 2.3, except that the annealing temperature was 55 °C. For visualizing the PCR fragments, 5 µL of the PCR product was used, and the remaining 10 µL was cleaned up with the ExoSAP protocol; 0.5 µL of exonuclease I (1 U/µL), 1 µL of shrimp alkaline phosphatase (20 U/µL), and 5 µL of PCR product were mixed and kept at 37 °C for 30 min, followed by 80 °C for 15 min for final inactivation of the enzymes. The subsequent sequence reaction was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the purification of sequence products was done with the BigDye XTerminator Purification Kit (Applied Biosystems). Briefly, 8–25 ng of PCR product, 2 µL of BigDye reaction mix, 2 µL of 5X Sequencing Buffer, and 1.2 pM primer were mixed and brought up to 10 µL with pure water. The sequencing PCR was initiated at first at 95 °C for 1 min, and then 35 cycles (95 °C 1 min, 50 °C 15 s, 60 °C 4 min) were performed. The final PCR products were cleaned up again, adding 40 µL of SAM solution and 10 µL of XTerminator solution from the purification kit and keeping it for 30 min at 2000 g. Finally, the mixtures were centrifuged at 1000 rpm for 2 min and loaded to the ABI Prism 3500 DNA Analyzer (Applied Biosystems).

2.5. Sequence analyses

The sequences obtained from the DNA analyzer were visualized using the software BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). In total, 127 PPV sequences, including the P3 and 6K1 regions of isolates deposited in GenBank, were used for the analysis. The list of GenBank isolates is provided in the Appendix. All sequences were aligned using BioEdit and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the sequences were trimmed to 664 bp to form clear consensus sequences. The number of changes at nucleated and aa levels were counted from the BioEdit alignment when all isolates belonging to a strain showed the change. The sequences were further analyzed using Mega 6 (Tamura et al., 2013), and the neighbor-joining method was used for phylogenetic tree construction, with a bootstrap value of 1000. Estimates of average evolutionary divergence over sequence pairs within groups and the intragroup genetic variability analyses were conducted using the maximum composite likelihood model (Tamura et al., 2004) implemented in MEGA 6. The novel sequences

obtained in this study have been deposited in the GenBank database and have been assigned the accession numbers KM409731–KM410044. The names of the isolates deposited in GenBank were made by combining the province name, district name, tree species, and order number in the collection. For example, KnMvAp326 stands for Konya (the province), Mevlana (the district in the province), apricot, and the collection number.

3. Results

3.1. Plum pox virus occurrence

In total, 612 samples were collected in 2012 and 2013. Samples were taken from either residential gardens or commercial orchards. The samples were collected from districts where PPV occurrence had been known previously. Of the total, 524 (86%) samples were identified as PPV-positive (Table 1). In most cases, symptomatic leaves were collected; thus, random sampling was not performed. Therefore, the rate of infected samples is not likely to represent the prevalence of PPV in the provinces. Indeed, we observed that most apricot trees in the Ortaköy district of Aksaray, in Ankara, in Kayseri, and in old districts of Konya and İstanbul exhibited PPV symptoms, but we did not collect samples from all the trees exhibiting symptoms of PPV. The numbers of positive samples in apricot samples in Kayseri, Ankara, Konya, the Ortaköy district of Aksaray, and İstanbul were found to be 88 (98%), 140 (97%), 57 (97%), 70 (96%), and 20 (80%), respectively. Although some plums exhibited PPV symptoms, many plums (as well as cherries and peaches) did not show obvious PPV symptoms. The number of positives samples in plum samples in Kayseri, Ankara, Konya, the Ortaköy district of Aksaray, and İstanbul were found to be 15 (79%), 13 (93%), 11 (73%), 12 (80%), and 6 (67%), respectively. The location, sample tree, number of infected samples, and infection rate for all samples are shown in Table 1. In Eskişehir, only four PPV symptom-showing apricots were found close to each other, and only one infected apricot tree in the Kırklareli city center was observed. Although sometimes leaves were also gathered from surrounding trees in these two provinces, PPV was detected only on symptom-showing trees in general. We did not observe symptoms in the city centers of Antalya, Balıkesir, Burdur, Bursa, Kırşehir, Malatya, Manisa, Nevşehir, Niğde, Sivas, Yalova, or Yozgat. In commercial orchards, samples were collected only from those orchards that were reported to have PPV symptoms based on information provided by local agricultural officers. PPV symptoms in apricot, plum, and peach orchards in Aydın, Bursa, Çanakkale, Denizli, İzmir, and Kayseri were identified. A map of Turkey presenting the PPV-infected districts is shown in Figure 1. Photos of fruits with symptoms were taken when infected fields were revisited (Figure 2).

3.2. Plum pox virus strain identification

Serologic and molecular typing was performed for strain identification (Table 2). For serological testing, DASI-ELISA was performed with commercially available antibodies only for the 215 samples of 2012. The values of the spectrophotometer ranged from –0.39 to 3.998 for antibody 5B-IVIA, which detects all strains of PPV, and 169 samples were accepted as positive. The average negative sample values ranged from –0.039 to 0.015 for antibody 5B-IVIA. Of the positive samples, 81 samples showed a positive reaction to the PPV-M strain-specific antibody. Twenty-eight samples reacted to both PPV-M- and PPV-D-specific antibodies. The samples from Aksaray identified as PPV-D by phylogenetic analysis interestingly did not react to the PPV-D-specific antibody. Spectrophotometric

Figure 1. Locations and strains of PPV isolates in Turkey.

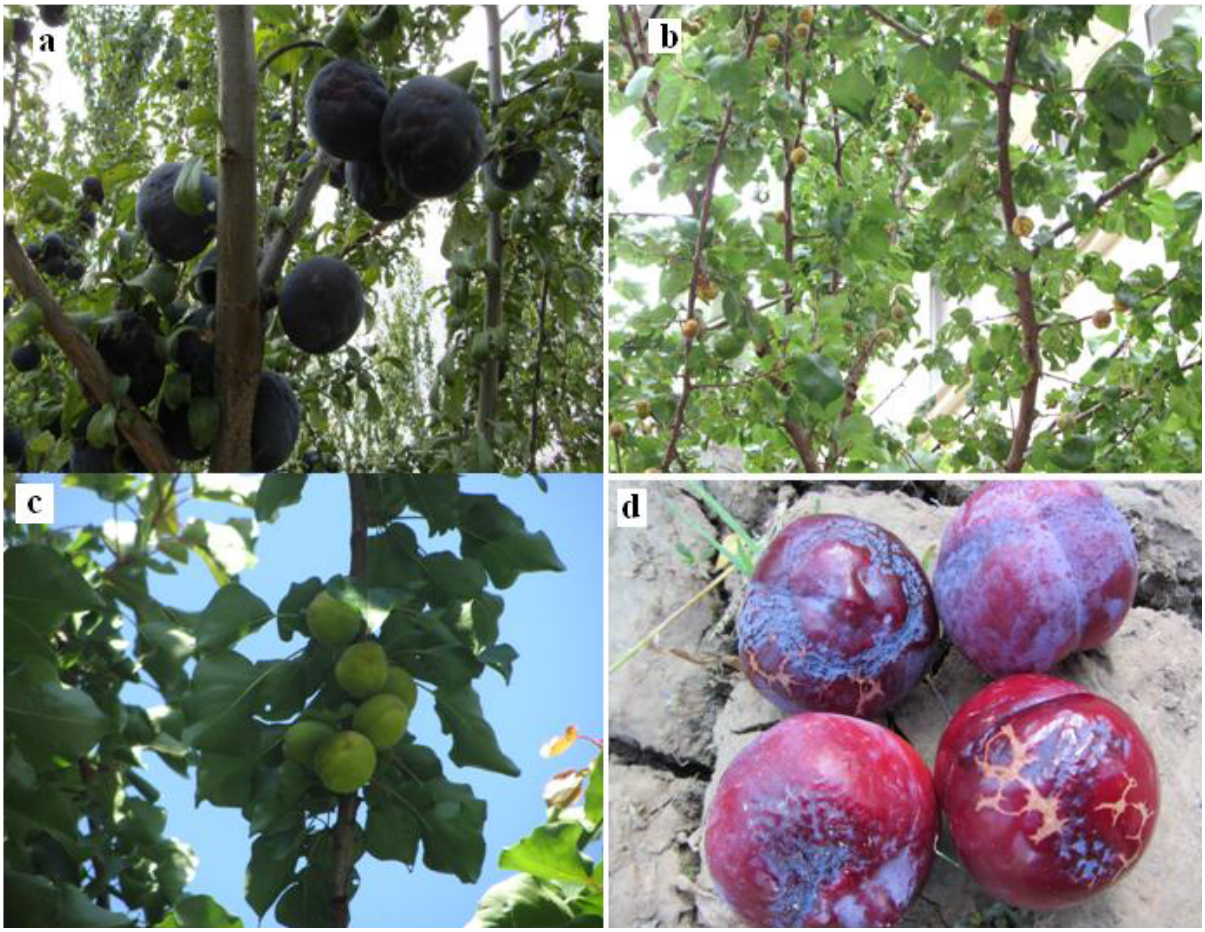

Figure 2. Symptoms caused by plum pox virus: a) PPV-M on a Black Diamond plum tree in Kayseri, b) PPV-D on an apricot seedling in Eskişehir, c) PPV-T on an apricot seedling in Kayseri, d) PPV-M on Autumn Giant plum fruit in Kayseri.

values for Aksaray samples ranged from –0.073 to 0.004, even though PPV-T samples exhibited good affinity for the 4D antibody ranging from 0.207 to 1.51, whereas negative sample values ranged from –0.085 to 0.010.

For molecular typing, RT-PCR with three primer pairs and sequence analysis was performed. Random-primed cDNA was constructed for all the samples, and PCR was performed using the available published primers: P1/P2 specific to all PPV strains, P1/PD specific to D, and P1/PM specific to PPV-M. The primers P1/P2, P1/PM, and P1/PD amplified 524, 513, and 89 samples, respectively. cDNA libraries were further amplified using a PP3/PCI primer pair to obtain an 836-bp amplification product representing the C-terminal of the P3 gene and the 6K1 gene of the PPV genome. A successful PCR product was obtained from 421 samples. PCR products were then sequenced using the PP3 primer. Good quality sequences were trimmed to obtain consensus. The consensus fragments included a 664-nt length spanning 511 nt of the C-terminal of the P3 gene and 153 nt of the 6K1 gene. A total of 314 sequences were obtained.

Although serological and PCR typing were performed for strain identification, reliable PPV typing was obtained from the sequencing analysis. This result happened mainly because there are no available monoclonal antibodies or PCR primers for the T strain. In addition to this disadvantage, a high proportion of isolates were identified as PPV-T in the Turkish PPV isolate pool by sequence analysis. Moreover, samples from Aksaray failed to bind D-specific antibodies. Thus, the ELISA produced confusing results. P1/PM primer pairs amplified all isolates as the PPV-universal P1/P2 primer did. Therefore, for an accurate interpretation of strains of isolates, we focused on sequence analysis. PPV-D, PPV-M, PPV-T, and Rec strains were identified in different districts of the country. Of the 314 sequenced isolates, 190 (60.51%), 84 (26.75%), 39 (12.42%), and 1 (0.32%) were identified as PPV-T, PPV-D, PPV-M, and PPV-Rec, respectively. Isolates of the Ortaköy district of Aksaray were identified as PPV-D. In Ankara, which is already known to be highly infected, 115 (94.26%) of the total isolates were identified as PPV-T and the rest as PPV-D. PPV-D isolates in Ankara

Table 2. Serological and molecular typing of detected PPV isolates. The table includes 314 samples that were demonstrated to be positive by sequence analysis. Serological tests were performed for only Kayseri and Aksaray samples. Molecular typing was done only on the CP gene, using P1/PD and P1/PM. PPV-D samples in Aksaray failed to react with PPV-D-specific antibody 4D.

	Sample		Serological			Molecular			
Source city	Number	Source field	PPV-all	PPV-M	PPV-D	PPV-all	PPV-M	PPV-D	Strains[1]
Aksaray	56	Residential	+	-	-	+	+	+	D
Ankara	115	Residential	NA	NA	NA	+	+	-	T
Ankara	7	Residential	NA	NA	NA	+	+	+	D
Aydın	8	Orchard	NA	NA	NA	+	+	-	M
Bursa	1	Orchard	NA	NA	NA	+	+	-	Rec
Bursa	3	Orchard	NA	NA	NA	+	+	-	D
Çanakkale	5	Orchard	NA	NA	NA	+	+	-	M
Denizli	2	Orchard	NA	NA	NA	+	+	-	M
Eskişehir	3	Residential	NA	NA	NA	+	+	-	D
Isparta	6	Orchard	NA	NA	NA	+	+	-	M
İstanbul	14	Residential	NA	NA	NA	+	+	-	M
İstanbul	6	Residential	NA	NA	NA	+	+	-	T
İstanbul	1	Residential	NA	NA	NA	+	+	+	D
İzmir	3	Orchard	NA	NA	NA	+	+	-	T
Kayseri	6	Residential	+	-	-	+	+	-	T
Kayseri	8	Residential	+	+	-	+	+	-	T
Kayseri	14	Residential	+	+	+	+	+	-	T
Kayseri	2	Orchard	+	+	-	+	+	-	M
Kayseri	1	Orchard	+	+	-	+	+	-	M
Konya	35	Residential	NA	NA	NA	+	+	-	T
Konya	9	Residential	NA	NA	NA	+	+	+	D
Kırklareli	1	Residential	NA	NA	NA	+	+	-	T
Tekirdağ	5	Residential	NA	NA	NA	+	+	+	D
Tekirdağ	1	Residential	NA	NA	NA	+	+	-	M
Tekirdağ	2	Residential	NA	NA	NA	+	+	-	T

NA: Not available. Serological tests were performed for only the 2012 collections.
[1]The strains were assumed based on sequence analysis and grouping of the phylogenetic analysis.

were only identified in the Çankaya district, but T samples were distributed over all neighborhoods of the city. Like Ankara, isolates of Konya were found to be PPV-D and PPV-T. Isolates of İstanbul and Tekirdağ were identified as PPV-D, PPV-M, and PPV-T strains. Residential trees in Kayseri and one apricot tree in Kırklareli were found to host PPV-T isolates. The positive samples of Eskişehir were identified as PPV-D. Isolates of orchards collected from Aydın, Çanakkale, Denizli, Isparta, and Kayseri were identified as the PPV-M strain, whereas an orchard in İzmir was found to host the PPV-T strain. However, orchards in Bursa were found to host PPV-D isolates, and only one isolate in Bursa was identified as PPV-Rec. This Rec isolate was the only strain identified in the country, other than PPV-D, PPV-M, and PPV-T strains.

3.3. Phylogenetic analysis based on the P3-6K1 region

Neighbor-joining phylogenetic analysis of P3-6K1 genes of 314 novel and 132 GenBank-deposited PPV isolates was performed using the method of maximum composite likelihood implemented in MEGA software. As a result of this analysis, Turkish isolates were grouped with the PPV-D, PPV-M, PPV-Rec, and PPV-T records of GenBank. The number of variable nucleotides and variable aa residues among the groups were counted directly after

obtaining consensus for each group of D, M, and T strains. The number of variable nucleotides among D, M, and T groups ranged from 5 to 91 (Table 3), whereas 12 variable aa positions were observed among M, D, and T isolates (Table 4).

All the PPV-T isolates collected in this study grouped with GenBank T records (Figure 3a). PPV-T isolates were grouped into two clusters on the dendrogram and named as Ta and Tb, although the two groups were not supported with a high bootstrap value (67%). However, two groupings were needed to explain the number of variable nucleotides (Table 3) and variable aa residues among the group of isolates (Table 4). Ta and Tb differed at nine nucleotides in the P3-6K1 region. At seven positions (3109, 3249, 3272, 3381, 3450, 3566, and 3572), Ta had nucleotides identical to the PPV-D isolates, whereas the PPV-Tb isolates were identical to the PPV-M isolates at the same seven of nine positions. Ta and Tb show three aa residue mutations, and Ta is identical to the D isolates for these three positions. Tb differs from Ma and Mb at only one position (1076) but not from the group of M İstanbul. PPV-Ta consisted mainly of samples collected from Ankara, Kayseri, and Konya. Ta isolates were not found in other regions where T isolates were identified. All isolates from Kayseri were grouped together, along with 10 samples from Ankara (Figure 3b). The PPV-Tb group showed more diversity, including isolates from Ankara, İstanbul, İzmir, Konya, Kırklareli, and Tekirdağ. Samples from Konya grouped together under PPV-Tb. The GenBank isolates Abtk, PI38, and PI45 placed in the Ta group, whereas AP39 was positioned in the Tb group. All of the PPV-M isolates collected from the orchards in Turkey dispersed among European PPV-M isolates (Figure 3c). However, 13 isolates of İstanbul formed a separate group adjacent to European PPV-M isolates. Previously, Dallot et al. (2011) suggested that PPV-M could be classified into two groups: PPV-Ma and PPV-Mb. Orchard PPV-M isolates did not show a consensus nucleotide difference from the European PPV-M isolates. In contrast, İstanbul M isolates differed at 8- and 10-nt positions from Ma and Mb, respectively. D isolates

Table 3. Number of variable nucleotides at the 664-nt length, spanning 511 nt of the C-terminal of the P3 gene and 153 nt of the 6K1 gene. Ta, Tb, Ma, and Mb include 2, 1, 17, and 15 GenBank records, respectively. The number of accessions analyzed per group is shown in parentheses.

Groups (number of isolates)	D GenBank (75)	D Turkey	D Aksaray	Ta	Tb	An	M Turkey	Ma
D Turkey (28)	11							
D Aksaray (58)	16	6						
Ta (127)	83	85	87					
Tb (66)	87	92	91	9				
An (1)	87	90	89	20	12			
M-İstanbul (13)	84	88	89	22	11	15		
Ma (42)	85	87	91	20	13	15	8	
Mb (16)	86	88	91	16	12	15	9	5

Table 4. Variable amino acid residues at the C-terminal of P3 and 6K1 regions of PPV groups.

Groups	963	980	986	988	1026	1055	1076	1079	1093	1102	1157	1164
D Turkey	G	N	R	V	L	L	S	V	R	H	I	A
D GenBank	G	N	R	V	L	L	S	V	G	H	I	A
REC	G	N	R	V	L	L	S	V	G	H	I	A
Ta	A	S	K	V	F	V	S	V	Q	H	V	T
Tb	A	S	K	A	F	V	S	I	Q	Y	V	T
An	A	S	K	A	F	V	S	I	Q	Y	V	T
M İstanbul	A	S	K	A	F	V	S	I	Q	Y	V	T
Mb	A	S	K	A	F	V	L	I	Q	Y	V	T
Ma	A	S	K	A	F	V	L	I	Q	Y	V	T

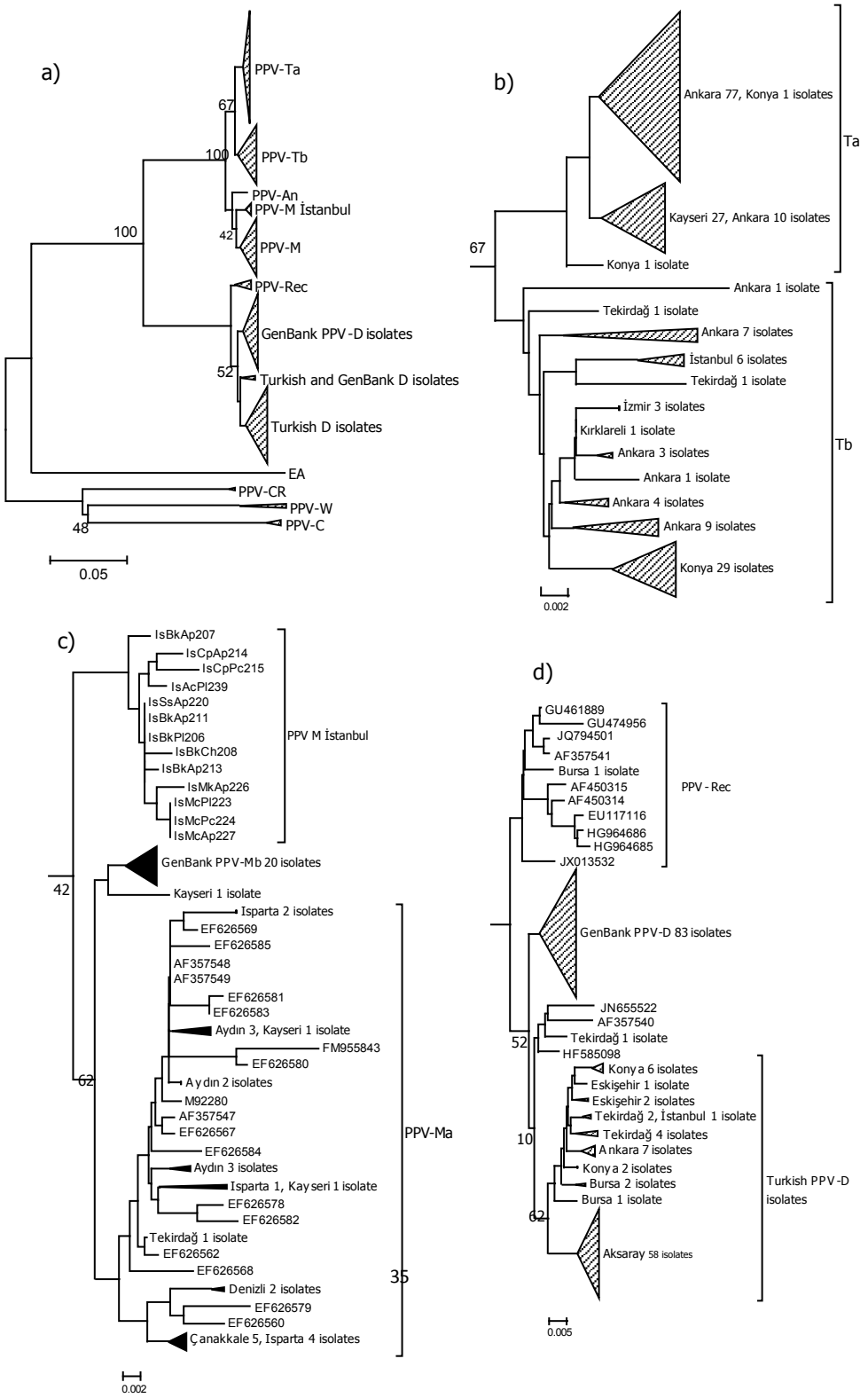

Figure 3. Neighbor-joining phylogenetic analysis view of the isolates. The accession numbers for the GenBank isolates in the compressed groups are given in the Appendix. a) The general view of the phylogenetic analysis of the nucleotide sequence of P3-6K1 genes of 314 novel and 127 GenBank-deposited PPV isolates. b) PPV-T group of the phylogenetic tree. c) PPV-M group of the phylogenetic tree. The upper 13 isolates are the M İstanbul isolates. d) PPV-Rec and PPV-D group of the phylogenetic tree. The upper 11 isolates are PPV-Rec.

in Turkey except only one sample grouped together on the phylogenetic tree with a low bootstrap value of 52% (Figure 3d). Samples from the each province partly clustered together, slightly reflecting their geographic separation. All isolates of Aksaray formed a group supported by a 62% bootstrap value, and they differed at 6 and 15 nt from the remaining D isolates of Turkey and GenBank D samples, respectively. The remaining D isolates (other than the isolates of Aksaray) in Turkey differed from GenBank D isolates by 11 nt. The D isolate of Aksaray differs from the global D isolate at 2 aa residue positions, whereas there is only one mutation difference between Turkish D isolates (except D isolates of Aksaray) and global D isolates.

The average evolutionary divergence over sequence pairs within groups was estimated for groups that had more than one GenBank record for the P3-6K1 region. The isolate numbers within groups are shown in Table 5. Overall, diversity in the PPV population was found to be 0.054 for the 446 PPV isolates. The mean intragroup diversity was the highest (0.049) for W. M, Rec, and T exhibited the same mean intragroup diversity, 0.017. C, D Turkey, D GenBank, and CR strains showed 0.14, 0.012, 0.009, and 0.007 intragroup diversity, respectively. The 13 M isolates, which were identified only in İstanbul, exhibited 0.005 intragroup diversity. Similarity over sequence pairs between groups was estimated (Table 6). PPV-T showed identity with An, M, Rec, D, EA, CR, W, and C at percentages of 96.9%, 96.3%, 85.8%, 85.1%, 69.2%, 68.1%, 65.3%, and 65.0%, respectively.

4. Discussion

PPV strains and variability in Turkey were studied; PPV infection was identified in Adana, Ankara, Aydın, Aksaray, Bursa, Çanakkale, Denizli, Isparta, İzmir, İstanbul, Kayseri, Konya, Kırklareli, and Tekirdağ. Since PPV was first reported in 1969, several studies have showed PPV occurrence in different locations: Ankara, Antalya, Aksaray, Edirne, Balıkesir, Bilecik, Bursa, Çanakkale, Kayseri, Konya, İzmir, İzmit, Manisa, Mersin, Sakarya, Samsun, Tekirdağ, and Yalova (Kurçman, 1973, Yürektürk, 1984; Dunez, 1986; Erdiller, 1988; Elibüyük and Erdiller, 1991; Azeri, 1994; Elibüyük, 2003, 2004, 2006; Koç and Baloğlu, 2006; Candresse et al., 2007; Gümüş et al., 2007; Çıtır and İlbağı, 2008; İlbağı et al., 2008; Akbaş et al., 2011; Ulubaş Serçe et al., 2011; Çelik and Topkaya Kütük, 2013; Ceylan et al., 2014; İlbağ and Çıtır, 2014; Deligöz et al., 2015). PPV in Aydın, Denizli, Eskişehir, İstanbul, and Kırklareli has been reported for the first time in the present study. Determining new infected locations suggests that the spread of PPV continues in the country.

Phylogenetic clustering and genetic similarity based on the P3-6K1 region of the 314 Turkish isolates enabled us to determine the strains of isolates in Turkey more precisely comparing ELISA test and PCR primers. This is mainly because currently there are no available monoclonal antibody and primer pairs to capture PPV-T isolates. Without sequencing, we could not distinguish PPV-T isolates from PPV-M isolates. Sequence analysis confirmed the existence of PPV-M and PPV-T strains in the Turkish isolate pool. However, it is worthwhile to mention that molecular typing with strain-specific primers is powerful even though no specific PPV-T primers are yet available. The sequences of PPV-T isolates obtained during this study should help in designing specific PPV-T primers. The PPV-T strain was identified in Ankara, İzmir, İstanbul, Kayseri, Konya, Kırklareli, and Tekirdağ and was found to be the dominant strain in the Turkish PPV pool. In previous studies, PPV-T isolates were reported

Table 5. Estimates of evolutionary similarity over sequence pairs between groups based on 664-nt length, spanning 511 nt of the C-terminal of the P3 gene and 153 nt of the 6K1 gene. The number of base substitutions per site from averaging over all sequence pairs between groups is shown. Standard error estimates are shown above the diagonal. The number of accessions analyzed per group is shown in parentheses.

Groups	T	An	M	REC	D	C	EA	CR	W
T (194)		0.005	0.005	0.014	0.015	0.033	0.031	0.034	0.032
An (1)	0.969		0.005	0.016	0.016	0.032	0.028	0.035	0.033
M (71)	0.963	0.974		0.015	0.016	0.032	0.027	0.033	0.031
REC (10)	0.858	0.853	0.856		0.005	0.034	0.032	0.037	0.033
D (159)	0.851	0.847	0.851	0.968		0.033	0.031	0.036	0.031
EA (1)	0.692	0.687	0.702	0.693	0.684		0.037	0.031	0.035
CR (3)	0.681	0.696	0.702	0.682	0.695	0.648		0.023	0.021
W (3)	0.653	0.655	0.658	0.649	0.660	0.666	0.770		0.028
C (4)	0.650	0.660	0.661	0.660	0.669	0.651	0.771	0.743	

in Ankara, İzmir, Tekirdağ, and Samsun (Ulubaş Serçe et al., 2011; İlbağ and Çıtır, 2014; Deligöz et al., 2015). Here, PPV-M isolates were identified in Aydın, Çanakkale, Denizli, İstanbul, and Isparta. In earlier research, PPV-M was identified in orchards of Adana (Koç and Baloğlu, 2006), Çanakkale, Mersin, Hatay (Ulubaş Serçe et al., 2011), the Yahyalı district of Kayseri (Ceylan et al., 2012), and Antalya (Çelik and Topkaya Kütük, 2013). In all these studies, PPV was identified in newly established peach or apricot orchards (usually 4–7 years old). Similarly, we identified PPV-M strains in young orchards of apricots in Aydın and Denizli, as well as in young orchards of peaches in Çanakkale and Isparta. We assume that these PPV-M isolates are likely newly introduced through illegal seedling traffic because it is known that peach and apricot orchards in coastal regions have been established with early ripening European breeding varieties rather than local varieties. The phylogenetic analysis of PPV-M isolates collected from orchards supported the idea that these M isolates could be introduced because they are distributed in the European PPV-M isolates' group on the dendrogram, without forming a separate group. However, PPV-M isolates in İstanbul that were collected from residential gardens formed a separate group on the phylogenetic tree, suggesting that M İstanbul isolates are not likely a recent introduction. PPV-M was also identified in Ankara by several studies (Elibüyük, 2003, 2004; Sertkaya et al., 2003) by mostly using PPV-M-specific monoclonal antibodies. We assume that PPV-T samples in Ankara were misidentified as PPV-M. The reason for misinterpretation was already revealed by previous studies reported by Boscia et al. (1997), Myrta et al. (1998), and Candresse et al. (1998). These researchers showed that PPV-T isolates reacted to some PPV-M-specific monoclonal antibodies. In our study, PPV-T isolates also reacted to the PPV-M-specific antibody AL. Some isolates collected in the residential gardens of the Ortaköy district of Aksaray, Bursa, Eskişehir, Konya, İstanbul, and Tekirdağ and in orchards of Bursa were identified as PPV-D. The occurrence of PPV-D was previously reported in Ankara (Elibüyük, 2004). Based on serological testing, that author reported that apricots and plums were infected by PPV-D at rates of 4.2% and 2.9%, respectively. It was also reported that apricots, plums, and peaches have mixed infection of PPV-M and PPV-D, with rates of 21.3%, 20.3%, and 6.2% in Ankara, respectively (Elibüyük, 2004). Our findings are partly compatible with reports of Elibüyük (2011). Based on sequence data, we also identified PPV-D infection in Ankara. However, we are cautious about the rates of mixed infection in Ankara. We assume that most of the mixed infections identified by Elibüyük (2011) could be PPV-T, similar to the misinterpretation of PPV-T as PPV-M, because T isolates could also react to PPV-D-specific antibodies (Boscia et al., 1997; Candresse et al., 1998; Myrta et al., 1998). Other than D isolates in Ankara, PPV-D occurrence has not been reported in other regions in Turkey. Therefore, the PPV-D strain in the Ortaköy district of Aksaray, Bursa, Eskişehir, Konya, İstanbul, and Tekirdağ was identified for the first time. In addition to PPV-M, -D, and -T strains, an isolate in Bursa was identified as PPV-Rec. The incidence of PPV-Rec was also reported once in Isparta in the country (Candresse et al., 2007). We did not collect many samples from Bursa. In a national-level survey, Akbaş et al. (2011) reported infection occurrence in different provinces and obtained the highest total infection in the province of Bursa, which is known as a main peach-producing location in the country. Moreover, Bursa is one of the main locations for nursery farming in the country, so the seedlings of varieties of European breeding programs are distributed from Bursa to the rest of the country. A more detailed survey in Bursa is needed to elucidate the prevalence of PV-Rec and other strains.

Nucleotide sequences for entire genomes and for informative genome portions, such as P3-6K1 and NIb-CP, are available in GenBank for PPV-M isolates prevalent throughout southern, eastern, and central Europe. Full genomes of PPV-D isolates obtained from many countries including the United States, Canada, and Japan, in addition to European countries, are also available in GenBank. Genomic information has also been produced for PPV-Rec, PPV-W, and PPV-CR (Matic et al., 2006; Thompson et al., 2009; Glasa et al., 2011; Sheveleva et al., 2012; Chirkov et al., 2013; Glasa et al., 2013; Mavrodieva et al., 2013). However, there was a limited genomic record of PPV-T, which was only detected in Turkey and Albania (Ulubaş Serçe et al., 2009; Palmisano et al., 2015), in GenBank. Moreover, there were no records of Turkish PPV-D and PPV-M sequences in GenBank. We have sequenced the P3-6K1 region of Turkish D and M isolates and T isolates. The sequences enabled us to determine the genetic variability of PPV-T, Turkish PPV-D, and PPV-M isolates, as well as to compare Turkish isolates with global PPV records. Relatively high intrastrain genetic variability was detected in Turkish PPV isolates. PPV-D in Turkey exhibited slightly higher genetic variability than global D isolates. Genetic variation in viruses is generated by mutation and recombination. Once a genetic change has occurred, the frequency of genetic variants in the population may be determined by several factors including drift and selection. Host-associated and vector-associated selections are major factors for explaining the frequency of genetic variants (García-Arenal et al., 2003). The aphid transmission nature of the virus in Turkey could be one of the explanations for higher genetic variation. After an infection occurs in a region, transmission of isolates probably happens by one tree with

aphids in residential home gardens because stone fruits in residential gardens are mostly not varieties but are grown from seeds. In clonally propagated orchards, trees originate from a limited number of bud donor trees. Because of that, PPV may be transferred long distances by seedlings and without showing much genetic diversity. However, if trees are propagated by seedlings, infection of each tree is likely to be a new occurrence by aphid transformation. Turkish PPV-D, PPV-M isolates of İstanbul, and PPV-T isolates grouped together on the phylogenetic tree. This can be interpreted as the demonstration of a long history for PPV-D, PPV-M isolates of İstanbul, and PPV-T strain in the country. Only PPV-M isolates collected from orchards dispersed into the group of European PPV-M isolates in the phylogenetic tree. This suggests that recent introductions of European M isolates have occurred or still continue to occur in the country.

PPV-T, Turkish D, and Turkish M strains showed subgroups on the phylogenetic tree, without supportive bootstrap values. However, there are some clues to consider them as candidates for future studies. For example, Ta and Tb differed at 9 nt, and at these positions, Ta had nucleotides identical to the PPV-D isolates, whereas the PPV-Tb isolates were identical to the PPV-M isolates. The PPV-M isolates from different provinces grouped with European PPV-M isolates, but only PPV-M isolates of residential trees in İstanbul formed a separate group. Grouping of Turkish D isolates is likely to reflect their collection geography. However, the subgroups of the strain were not supported with reliable bootstrap values, and limited genomic regions were investigated in this study. Therefore, the subgrouping in Turkish isolates and depiction of the subgroups on the phylogenetic trees in Figure 3 was performed with an intention of better expressing the genetic relations among the Turkish isolates and providing clues for further investigations, such as full genome sequencing or biological characterization.

In conclusion, determining the new occurrence of PPV in some provinces suggested that PPV continues to spread in the country. A high prevalence of PPV infections was observed in the city centers of İstanbul and Konya, and in Ortaköy in Aksaray, in addition to Ankara and Kayseri, both of which were already known to have high prevalence rates of infection. PPV-T and PPV-D strains were mostly identified in residential gardens in city centers. However, European PPV-M isolates were identified in orchards of Aydın, Çanakkale, Denizli, Isparta, and Kayseri. This supported the previous reports that new orchards were infected with the PPV-M strain, especially in the coastal region, and phylogenetic analysis demonstrated that M isolates in orchards could be new introductions in Turkey. However, like PPV-T isolates, Turkish PPV-D and PPV-M isolates of İstanbul could have been present in the country for a long time. Full genome sequences of more samples may reveal more about the spread and history of the virus in the country. The increase in the spread of the virus in the country and its high variability are potential threats to the stone fruit industry.

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Appendix. List of GenBank isolates.

Isolate	Accession No.	Strain	Isolate	Accession No.	Strain	Isolate	Accession No.	Strain
Al11Pl	HF674399	An	93.08	AF357546	D	RS1	EF626570	M
SoC	AY184478	C	85.001	AF357550	D	RS2	EF626571	M
BY101	HQ840517	C	94.055	AF357551	D	RS3	EF626572	M
BY181	HQ840518	C	PENN1	AF401295	D	RS4	EF626573	M
SwC	Y09851.2	C	PENN2	AF401296	D	RS5	EF626574	M
RU-17sc	KC020124	CR	Fantasia	AY912056	D	RS6	EF626575	M
RU-18sc	KC020125	CR	Vulcan	AY912057	D	SK1	EF626576	M
RU-30sc	KC020126	CR	48-922	AY912058	D	SK2	EF626577	M
Ou1	AB545926	D	Cdn1	AY953261	D	GR2	EF626578	M
Ak1	AB576045	D	Cdn3	AY953262	D	GR1	EF626580	M
Ak2	AB576046	D	Cdn4	AY953263	D	IT1	EF626581	M
Ak3	AB576047	D	Cdn5	AY953264	D	IT2	EF626582	M
Ha1	AB576048	D	Cdn7-2	AY953265	D	IT3	EF626583	M
Ha2	AB576049	D	Cdn12	AY953266	D	CY1	EF626584	M
Ha3	AB576050	D	123-1	AY953267	D	CY2	EF626585	M
Ha4	AB576051	D	NA*	D13751	D	GR0019	FM955843	M
Hi1	AB576052	D	NAT	D13751	D	VAR-2/531	HF585100	M
Hi2	AB576053	D	Penn3	DQ465242	D	VAR-2/551	HF585101	M
Hi3	AB576054	D	Penn4cds4	DQ465243	D	VAR-2/SE	HF585102	M
Hi4	AB576055	D	Penn3cds1	EF611241	D	VAR-2/M13	HF585103	M
Ok1	AB576056	D	Penn3cds2	EF611242	D	VAR-2/B23	HF585104	M
Ok2	AB576057	D	Penn3cds4	EF611243	D	SK68	M92280	M
Ou2	AB576058	D	Penn3cds3	EF611244	D	BOR-3	AF357541	Rec
Ou3	AB576059	D	Penn4	EF611245	D	KRN-1	AF450314	Rec
Ou4	AB576060	D	Penn4cds3	EF611247	D	MYV-1	AF450315	Rec
Ou5	AB576061	D	Penn4cds1	EF611248	D	J4c	EU117116	Rec
Ou6	AB576062	D	Penn5	EF640933	D	BULG	GU461889	Rec
Ou7	AB576063	D	Penn6	EF640934	D	o6	GU474956	Rec
Ou8	AB576064	D	Penn7	EF640935	D	Cdn08	HG964685	Rec
Ou9	AB576065	D	Penn8	EF640936	D	Cdn10	HG964686	Rec
Ou12	AB576066	D	Penn9	EF640937	D	Valjevka	JX013532	Rec
Ou13	AB576067	D	Penn10	EF640938	D	Abtk	EU734794	T
Ou14	AB576068	D	Penn12	EF640939	D	AP39	EU734797	T
Ou15	AB576069	D	SK-272	HF585098	D	PI38	EU734799	T
Ou16	AB576070	D	DF2	JN655522	D	W3174	AY912055	W
Ou17	AB576071	D	NA*	KP998124	D	LV-145bt	HQ670748	W
Ou18	AB576072	D	NA*	AM157175	EA	UKR 44189	JN637992	W
Mi1	AB576073	D	94.061	AF357547	M			
Mi2	AB576074	D	91.003	AF357548	M			
Mi3	AB576075	D	91.006	AF357549	M			
Od1	AB576076	D	PS	AJ243957	M			
Od2	AB576077	D	BG1	EF626559	M			
Od3	AB576078	D	BG6	EF626564	M			
Od4	AB576079	D	CZ2	EF626565	M			
Od5	AB576080	D	CZ1	EF626566	M			
BIII2	AF357540	D	FR2	EF626567	M			
92.011	AF357545	D	FR4	EF626569	M			

*Not available