

Detection and Characterization of Plum Pox Potyvirus (PPV) by DAS-ELISA and RT-PCR / RFLP Analysis in Turkey

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Abstract: This study was conducted to determine the presence of plum pox virus (PPV) (family *Potyviridae*, genus *Potyvirus*) in different regions of Turkey and to characterize PPV isolates by serological and molecular techniques, including ELISA and PCR/ RFLP. Thus, leaf samples from different stone fruit species (almond, apricot, nectarine, peach, plum and sour and sweet cherry) exhibiting various types of symptoms related to PPV were collected from different parts of the canopy from randomly selected orchards in the main stone fruit growing areas in Turkey, except for Malatya province. Polyclonal antibodies were used to detect the presence of PPV in the plant samples by serological assays (DAS-ELISA). The following monoclonal antibodies (MAbs); Mab 5B (Universal), Mab 4DG5 (PPV-D: Dideron-specific), Mab AL (PPV-M: Marcus-specific), Mab EA24 (PPV-EI Amar-specific) and Mab AC (PPV-C: Cherry-specific), were used to identify the serotyping of PPV isolates. Reverse transcription-polymerase chain reaction (RT-PCR) assays and restriction fragment length polymorphism analysis of RT-PCR products were performed to characterize Turkish PPV isolates. The results of RT-PCR analyses using general primers were in complete agreement with the DAS-ELISA and DAS-ELISA results, showing that 2 of 52 stone fruit samples collected from apricots in Ankara province were infected with the M strain of PPV. This study confirmed the results of the previous work and demonstrated the presence of the PPV-M strain in apricots in Turkey.

Key Words: Enzyme-linked immunosorbent assay, plum pox potyvirus, restriction analysis, reverse transcription-polymerase chain reaction, sharka

Türkiye'de Plum Pox (Şarka) Virüsünün DAS-ELISA ve RT-PCR/RFLP Analizi ile Saptanması ve Tanılanması

Özet: Ülkemizin farklı bölgelerinde Plum pox virüs (PPV) (family *Potyviridae*, genus *Potyvirus*)'ünün belirlenmesi ve gelişmiş yöntemler ile PPV izolatlarının araştırılması amacı ile, Malatya yöresi dışında kalan ülkemizin önemli yetiştirici bölgelerinde tesadüfi olarak seçilen bahçelerden PPV'ye özgü belirtiler gösteren badem, erik, kayısı, kiraz, nektarin, şeftali, ve vişne ağaçlarından yaprak örnekleri toplanmıştır. Serolojik (DAS-ELISA) çalışmalarda polyclonal antikorlar ve monoclonal antikorlar (MAbs); Mab5B (Universal), Mab4DG5 (PPV-D: Dideron), MabAL (PPV-M: Marcus), MabEA24 (PPV-EI Amar) ve MabAC (PPV-C: Cherry) kullanılmıştır. Türkiye'ye ait PPV izolatlarını karakterize etmek için RT-PCR (Reverse transcription-polymerase chain reaction) / RFLP (Restriction fragment length polymorphism) analizi yapılmıştır. RT-PCR/RFLP analizi ile DAS-ELISA ve DAS-ELISA bulgularını destekleyen sonuçlar elde edilmiştir. Toplam 52 örnekten iki adetinin PPV ile enfekteli olduğu ve bu iki kayısı izolatının M straini olduğu belirlenmiştir. Bu çalışma ile Türkiye'de kaysıda PPV-M irkinin varlığına dair önceki araştırma bulguları desteklenmiştir.

Anahtar Sözcükler: ELISA, plum pox potyvirus, RT-PCR, RFLP analizi, şarka

Introduction

Stone fruits are of economic importance to Turkey and the annual production of apricots is 500,000 tons, of peaches and nectarines 400,000 tons, of cherries 200,000 tons, and of plums 180,000 tons (FAO, 2002). Plum pox potyvirus (PPV), the causal agent of Sharka disease, is considered the most devastating viral disease of stone fruit crops due to reduction in fruit quality, premature dropping of fruit, a wide host range in

cultivated and wild *Prunus* spp., its rapid natural spread by aphid vectors and the rapid decline and death of trees when co-infected with other viruses (Nemeth, 1986). A number of economically important stone fruit species, including the almond, apricot, nectarine, peach, plum and sour and sweet cherry, are known to be its hosts. Four major types of PPV have been identified based on symptoms, serological and epidemiological characteristics and comparisons at the protein and nucleic acid levels.

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Strain D (Dideron) was first time isolated from an apricot tree in Southeastern France and is the most common strain in apricots and plums in Western Europe and several Mediterranean areas. Strain M (Marcus) was isolated from a peach orchard in Northern Greece and occurs mostly in Southern and Eastern Europe (Pasquini and Barba, 1997; Myrta et al., 1998). These 2 strains, originally described by Kerlan and Dunez (1979), are the most important from an economic point of view because of their rapid spread by aphid vectors. Strain El Amar (EA) is represented only by an Egyptian apricot isolate and was differentiated initially in 1991 (Wetzel et al., 1991). The cherry strain (C) was first found in sour cherry trees in Moldavia and was characterized by Nemchinov and Hadidi (1996), and a sweet cherry isolate of PPV was investigated in Southern Italy and characterized by Crescenzi et al. (1997).

The presence of PPV in Turkey was reported from the Marmara region and Central Anatolia based on symptomatological observations in the surveys of 1968 and 1973 (Sahtiyancı, 1968; Kurçman, 1973). Several surveys have taken place in these regions during the last 20 years, and samples were examined by biological indexing and ELISA using polyclonal antibodies (PAbs) (Yürektürk, 1984; Elibüyük and Erdiller, 1991). Recently, many surveys were carried out in various stone fruit growing areas in Turkey. The Eastern Mediterranean region of Turkey was found to be free of PPV (Çağlayan and Gazel, 1997). The eastern part of Turkey, especially Malatya province, which is the most important apricot production area, has been surveyed and 1019 samples serologically tested. However, no PPV-infection on the apricot trees tested was determined by ELISA studies (Sipahioğlu, 1999; Yorgancı et al., 2001).

Although some research has been completed to characterize Turkish PPV isolates by using monoclonal antibodies (MAbs) in DAS-ELISA (Boscia et al., 1997; Candresse et al., 1998), more information is needed to clarify types of isolates in different regions. An ambiguous result (the presence of both M- and D-specific epitopes in the same isolate) was reported by Candresse et al. (1998) on a Turkish PPV peach isolate. Myrta et al. (1998) similarly reported that apricot samples collected from Turkey reacted with both the MAbs AL (M-specific) and 4DG5 (D-specific), and they suggested that further investigations on Turkish PPV isolates were necessary. Although some work regarding the characterization of Turkish PPV isolates has been done, detailed information is needed to find new PPV-infected samples and to clarify isolate types from different regions. This study was performed to determine the presence of PPV in different regions of Turkey and to characterize PPV isolates by serological (DAS-ELISA and DAS-ELISA) and molecular (RT-PCR/RFLP) techniques.

Materials and Methods

Source of the Plant Samples: Almond, apricot, nectarine, peach, plum and sour and sweet cherry leaves exhibiting various types of PPV-like symptoms were collected from different parts of the canopy from randomly selected orchards in the main stone fruit growing areas in Turkey, except for Malatya, in May, 2002. A total of 52 samples were collected from Adana, Hatay and Kahramanmaraş provinces in the Eastern Mediterranean region, Ankara province in Central Anatolia, İzmir in the Aegean region, Yalova in the Marmara region and Tokat in the Central Black Sea region (Table 1), (Figure 1).

Table 1. Number of stone fruit samples collected from different regions of Turkey in 2002.

Sample Collection Regions*	Stone Fruit Species							Total
	Almond	Apricot	Sour Cherry	Sweet Cherry	Nectarine	Peach	Plum	
CA		2				2		4
EMR	2	4	6	11	2	2	11	38
ER		2				2		4
CBSR		2						2
MR		2				2		4
Total	2	12	6	11	2	8	11	52

* CA: Central Anatolia, EMR: Eastern Mediterranean Region, ER: Aegean Region, CBSR: Central Black Sea Region, MR: Marmara Region

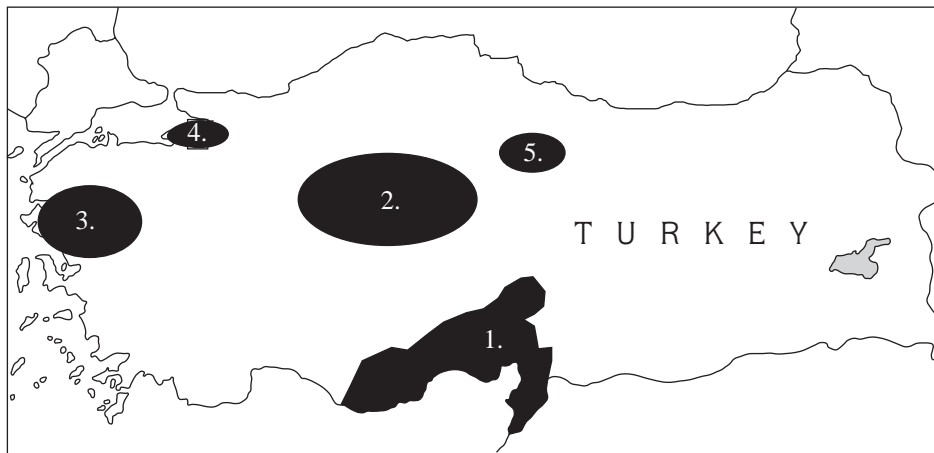


Figure 1. The sample collection and main stone fruit growing regions of Turkey:

1. Adana, Hatay and Kahramanmaraş provinces (Eastern Mediterranean region); 2. Ankara province (Central Anatolia); 3. İzmir province (Aegean region); 4. Yalova province (Marmara region); 5. Tokat province (Central Black Sea region).

Detection and characterization of PPV isolates by ELISA: Detection of PPV was performed according to the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) method described by Clark and Adams (1977) using commercial PABs. The ELISA reagents were supplied by Bioreba AG Chr. Merian-Ring 7 Reinach BL 1-Switzerland. The samples were prepared from leaves by homogenizing 1 g of tissues in extraction buffer (phosphate-buffered saline, pH 7.2 with 0.05% Tween 20, 2% polyvinylpyrrolidone and 0.2% ovalbumin) in a ratio of 1:10 and further diluted if necessary. The absorbance values were read on a Sirio S colorimeter at 405 nm. The samples were tested by DAS-ELISA in duplicate. Sensitivities were deduced from a detection threshold equal to twice the mean value of a healthy control. Leaves of PPV-M-infected and healthy GF 305 peach seedlings were used as positive and negative controls.

For the serotyping of PPV isolates, isolated from stone fruits, 5 PPV-specific MABs were used. The serotyping of PPV isolates was performed according to the previously described indirect DAS-ELISA (DASI-ELISA) method (Cambra et al., 1991), and the following monoclonals were used: Mab 5B (Universal), Mab 4DG5 (PPV-D specific), Mab AL (PPV-M specific), Mab EA24 (PPV-EI Amar specific) and Mab AC (PPV-C specific), which are mostly used for the detection of PPV strains in Europe (Cambra et al., 1994; Boscia et al., 1997; Myrta et al., 1998). Alkaline phosphatase enzyme-conjugated

goat anti-mouse immunoglobulins (Sigma GmbH, Germany) were used. The leaves of GF-305 peach seedlings inoculated with the PPV serotypes were used as positive controls for each serotype of PPV.

Isolation of RNA: Total RNA was extracted from 100 mg fresh and an equivalent amount of CaCl₂-dried leaves according to the LiCl method as described by Spiegel et al. (1996). For this purpose, plant material was homogenized by mortar and pestle with 5 volumes of buffer (200 mM Tris-HCl pH 8.5, 1.5% SDS, 300 mM lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, 1% igePAL CA-630) and 0.5% 2-mercaptoethanol). The extract was transferred into a 1.5 ml eppendorf tube. Following incubation at 65 °C for 15 min, an equal volume of 6 M potassium acetate, pH 6.5, was added and maintained on ice for 15 min. After centrifugation at 14,000 rpm for 10 min, nucleic acids were precipitated from the supernatant with isopropanol and centrifuged as above. The pellet was resuspended after drying in 50 µl sterile water. The leaves of GF-305 peach seedlings inoculated with PPV serotypes were used as reference isolates.

Detection and characterization of PPV isolates by PCR: For the general detection of PPV, universal primers located near the 3' end of the coat protein gene were used to amplify a 243 bp fragment (Wetzel et al., 1991). In order to differentiate PPV strains, the following PPV strain specific primer sets (Szemes et al., 2001) were

used: GCAGCAACTAGCCCAATAMT (D-5') and TGTTCCAAAAGTTTGCRRTTGAGGT (D-3') with an expected 159 bp product; The PPV-M specific primer pair GYGCAACRACCTCAACCAG (M-5') and CCTTCCTGYRTTCACCAAAGT (M-3'), amplify a 207 bp product. Primers TAGTCACCACTACACAGCAG (EA-5') and AGGAGGTGTAGTAGTTGTTG (EA-3') are PPV-EA specific, while GGGAAATGATGACGACGTA ACTCT (Ch-5') and CAATTACCCCATACGAGAAT (Ch-3') are specific for PPV-Ch and amplify a 167 and 224 bp product, respectively.

RT-PCR Reaction: The reverse transcription (RT) and PCR assays were performed in an interrupted thermal cycling program (Promega). PCR products were amplified in a 50 ml volume containing 1 ml of RNA, 20 pmol/ml of each primer, 100 mM of each dNTP's, 1 U Taq DNA polymerase (Promega), 1X reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1 mM MgCl₂ and Triton X-100) and 1.5 U AMV reverse transcriptase (Amersham). RT-PCR conditions were 30 min at 42 °C and 3 min at 93 °C, followed by 40 cycles at 94 °C, 30 sec at 54 °C and 30 sec at 72 °C with a final extension of 5 min at 72 °C. RT-PCR products were separated on a 2% agarose gel electrophoresis and visualized and photographed using a Polaroid gel camera system under UV after ethidium bromide staining.

PCR/RFLP Analysis: For restriction fragment length polymorphism (RFLP) analysis of the 243 bp fragment RT-PCR amplified products were incubated with 5 units of *RsaI* or *AluI* overnight at 37 °C. Digested products were analyzed by gel electrophoresis, then stained and photographed as above.

Results

Detection and characterization of PPV isolates by DAS-ELISA: Only 2 apricot samples collected from Ankara province reacted positively with the PPV specific polyclonal antibody in DAS-ELISA (Table 2). These 2 samples were also positive with PPV-M specific MabAL. The remaining 50 isolates did not react with any of the Mabs tested in this study. The averages of absorbance values of DASI-ELISA were given in Table 3.

Detection and characterization of PPV isolates by RT-PCR/RFLP: All stone fruit tree samples were tested by RT-PCR with the universal primers, with only 2 apricot samples from Ankara definitely reacting positive (Figure

Table 2. Detection of PPV by DAS-ELISA in stone fruit samples collected from different regions of Turkey in 2002.

Stone Fruit Species	No. of PPV-infected Samples	
	No. of Tested Samples	
Almond	0/2	
Apricot	2/12	
Sour Cherry	0/6	
Sweet Cherry	0/11	
Nectarine	0/2	
Peach	0/8	
Plum	0/11	
Total	52	

2) and 12 samples reacting borderline positive. The 2 PPV-positive apricot and the borderline samples were re-tested by RT-PCR and only two apricot samples were found PPV-positive.

Two positive PCR products with an expected size of 243 bp were analyzed by cleaving with *AluI* and *RsaI*. Both of the RT-PCR amplicons from 2 apricot isolates were cut by *AluI*, but not with *RsaI* (Figure 3).

To improve these data and test for other strains, RT-PCR was performed with strain specific primers of PPV (Figure 4). The PPV-M strain specific primers amplified 207 bp products, revealing that both apricot PPV isolates are members of strain-M. PPV-D and PPV-C strain specific primers did not produce any amplicon, except for the positive control of each strain. The PPV-EA-specific primers produced a weak band 167 bp in length along with other unspecified bands. Repeated analyses of the test gave the same results.

Discussion

PPV-infection was only detected from 2 apricot samples collected from Ankara province where the presence of PPV-infection has been recorded previously (Kurçman, 1973). Two PPV isolates from Ankara province gave very weak reactions only with MabAL, suggesting that PPV isolates are members of M serotype. Boscia et al. (1997) reported that Turkish isolates reacted positively with both MAbS specific to PPV-D and PPV-M. An interesting result was reported by Candresse et al. (1998) on a Turkish apricot isolate, inoculated on a GF 305 peach seedling. Named isolate Abricotier Turquie, this was the only isolate of the M serotype that did not

Table 3. Average of absorbance values (405 nm) of DASI-ELISA in stone fruit samples against monoclonal antibodies (MAbs) of plum pox virus (PPV).

Stone Fruit Species	Monoclonal Antibodies (MAbs)**				
	Mab 5B	Mab 4DG5	Mab AL	Mab EA24	Mab AC
Almond	0.170	0.182	0.160	0.148	0.154
Apricot	0.144	0.146	0.152	0.142	0.160
Sour Cherry	0.184	0.174	0.184	0.176	0.180
Sweet Cherry	0.178	0.162	0.166	0.182	0.172
Nectarine	0.172	0.158	0.164	0.168	0.158
Peach	0.182	0.166	0.186	0.158	0.162
Plum	0.144	0.154	0.156	0.148	0.148
Positive Apricot Samples*	0.514	0.198	0.628	0.150	0.154
	0.980		0.876		
Positive Control GF 305 peach		0.964	1.442	0.660	0.422
Negative Control GF 305 peach	0.170	0.178	0.144	0.174	0.168

* Absorbance values of PPV-infected apricot samples coded apricot1 and apricot2, respectively.

** Mab 5B: Universal; Mab 4DG5: PPV-D (Dideron-specific); Mab AL: PPV-M (Marcus-specific); Mab EA24: PPV-EI Amar (EI Amar-specific) and Mab AC: PPV-C (Cherry-specific).

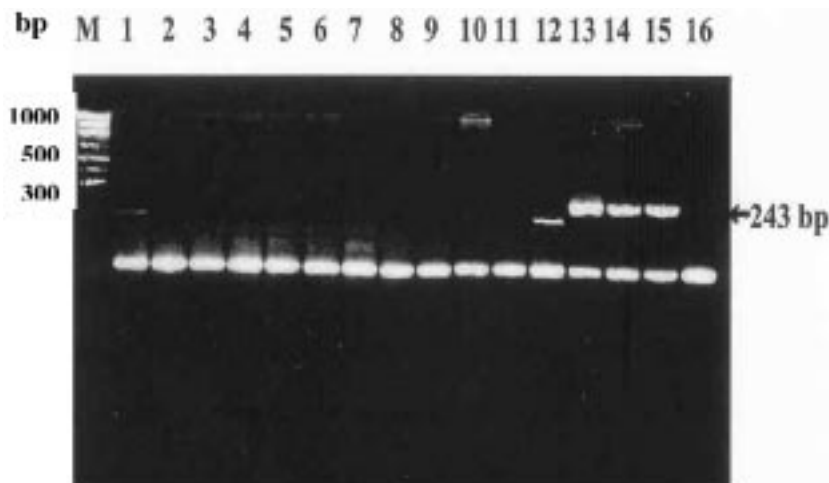


Figure 2. RT-PCR analysis of samples collected from Hatay and Ankara provinces. The arrow points to the expected products of the 243 bp fragment. DNA size markers (Boehringer) (Lane M); plum samples (Lanes 1,2,7,8 and 12); peach samples (Lanes 3-6 and 11); apricot samples (Lanes 10,13 and 14); positive control, GF-305 infected with PPV-M (Lane 15); H₂O control (Lane 16).

react with the MabAL, and it was suggested that Turkish isolates should be considered as members of the M serotypes based on PCR, but possessing both D- and M-specific epitopes. Myrta et al., (1998) also reported that PPV-M isolates that also contain D-specific epitope from apricot samples had been detected in Turkey. However, it was suggested that further investigation on Turkish PPV isolates is necessary.

The results of RT-PCR tests using general primers were in complete agreement with the DAS-ELISA and DASI-ELISA results. RT-PCR products 243 bp in size were produced from only 2 apricot isolates from Ankara province using the universal PPV primers (Figure 2). The isolates showing a faint 243 bp band (Figure 4) were re-tested by RT-PCR and were found to be negative, suggesting that they were artefacts. However, 2 apricot

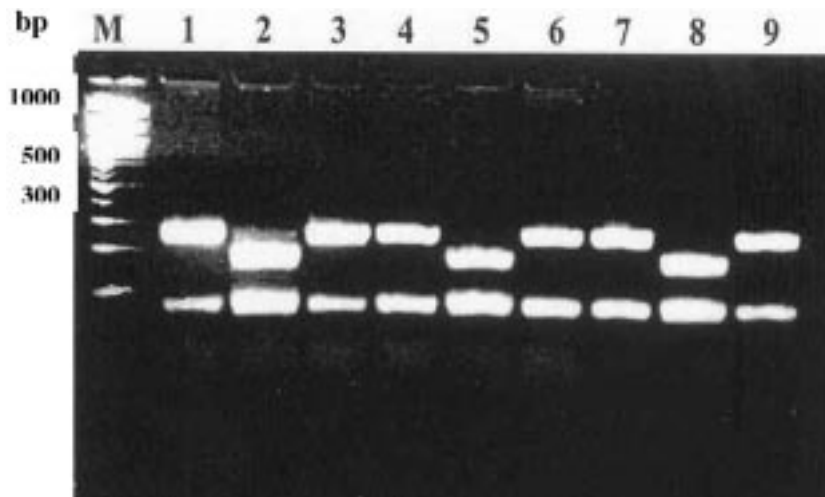


Figure 3. Restriction fragment length polymorphism (RFLP) analysis of 2 PPV apricot isolates (apricot1 and apricot2) by *AluI* and *RsaI*. DNA size markers (Boehringer) (Lane M); *RsaI*-digested RT-PCR products (Lanes 1,4 and 7); *AluI*-digested RT-PCR products (Lanes 2,5 and 8); undigested RT-PCR products (Lanes 3, 6, and 9). Lanes 1-3 are apricot1; Lanes 4-6 are apricot2; Lanes 7-9 are the PPV-M strain-positive control.

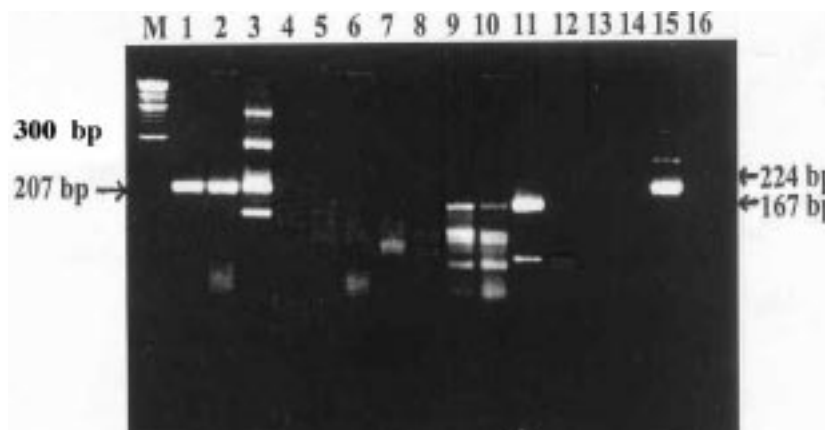


Figure 4. RT-PCR analysis of 2 PPV apricot isolates by PPV strain specific primers. DNA size markers (Boehringer) (Lane M); apricot1 (Lanes 1,5,9 and 13); apricot2 (Lanes 2,6,10 and 14); PPV-M strain-positive control (Lane 3); PPV-D strain-positive control (Lane 7); PPV-EA strain-positive control (Lane 11); PPV-C strain-positive control (Lane 15); H₂O control (Lanes 4,8,12 and 16).

isolates from Ankara province produced a 243 bp product with the universal PPV primer set in the repeated test.

RFLP analysis of PCR fragments could be used to discriminate between the D and M serotypes of PPV. PPV-M does not contain the *RsaI* recognition site, found in PPV-D (Wetzel et al. 1991; Candresse et al., 1994). The analysis of the amplified fragment by digestion by

AluI and *RsaI* revealed the presence of *AluI* restriction sites at the expected positions. The restriction analysis confirmed that the 2 PCR fragments contained an *AluI* site (Figure 3). RT-PCR/RFLP analysis showed that the 2 isolates are members of the M strain of PPV.

In order to test whether the PPV isolates were Cherry or El Amar strain, RT PCR analyses were performed

using C- and EA-strain specific primers in addition to the M- and D-strain specific primers. While PPV-M strain specific primers amplified a fragment of expected size, PPV-D and PPV-C strain specific primers only amplified the positive controls (Figure 4). These results support the findings of Boscia et al. (1997). Candresse et al., (1998) reported that although Turkish isolates reacted with both D- and M-specific monoclonal antisera, PCR analysis revealed only the presence of an M-serotype isolate. In our work, both of the apricot samples collected from Ankara were found to be infected with the M strain of PPV.

Although many suspicious samples from the point of view of PPV were collected from different provinces, not many infected samples were found in this study. Neither PPV-M nor PPV-D serotypes were found in peach samples from different locations from which PPV isolates have been reported (Candresse et al., 1998). It was suggested that PPV-infected trees had been killed by viruses or growers. In order to describe Turkish PPV isolates clearly, we believe that more samples from stone fruit

orchards in all growing locations need to be examined in further studies. Furthermore, PPV-infected trees, especially those with the PPV-M and D strains, must be eradicated as soon as possible due to the possibility of the strains rapidly spreading by aphid vectors in nature (Pasquini and Barba, 1997). Investigations need to be continued to determine new Turkish isolates on different stone fruit species from different locations to clarify the current situation of PPV in Turkey.

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