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Prunus serrulata is A Novel Natural Host of *Plum pox virus* in Türkiye

Havva ILBAGI[1*], Busra BAS[2], Ahmet CITIR[1]

[1]*Department of Plant Protection, Faculty of Agriculture, Tekirdag Namik Kemal University, 59030, Tekirdag, Türkiye*
[2]*Ferrero Degerli Agriculture Company, Sakarya, Türkiye*

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ABSTRACT

Japanese flowering cherry (*Prunus serrulata* Lindl. cv. Kanzan) tree is one of the most popular ornamental plant species with light pink double flowers having unfolded petals from crimson buds. It is recommended for landscape architecture and gardening arrangements because of its mass floral display in spring. *P. serrulata* Kanzan is grown as shade trees in home gardens, parks, and on the roadsides in the Trakya region as well as in Tekirdağ province of Türkiye. In 2019, some systemic disease symptoms such as mild diffuse chlorotic mosaic and slightly chlorotic local spots on leaves of some *P. serrulata* trees in Tekirdağ have been observed. To identify PPV, 15 symptomatic and asymptomatic leaf samples were collected from different *P. serrulata* trees growing on the roadside and in-home gardens, and screened by RT-PCR test. Two out of the fifteen samples were found infected with *Plum pox virus* (PPV). The amplicon of one positive sample was sequenced, and a partial sequence was submitted in the GenBank. Analysis of the obtained sequence revealed that it had 100-44.30% nucleotide and 100-10.97% amino acid identities of PPV isolates deposited in the GenBank from diverse origins of *Prunus* species worldwide. PPV is a causal agent of Sharka disease on stone fruits and ornamental *Prunus* trees. Until now, no information has been available about the occurrence as a natural host of PPV on *P. serrulata* Kanzan in Türkiye. To our best knowledge, this is the first report of PPV on *P. serrulata* Kanzan in Türkiye.

1. Introduction

The Trakya region of Türkiye includes Edirne, Kırklareli, and Tekirdağ provinces, which harbor many natural, horticultural, and ornamental tree species. There are at least 36 needle-leaved and 117 broad-leaved tree species in orchards, forests, parks, and arboretums as well as along the highways and on both sides of streets and other recreational areas in Trakya. Public and private nurseries produce the nurses of all those tree species to establish new recreational areas [1]. Besides stone fruits of apricot (*Prunus armenica* L.), peach (*P. persica* L. Batsch), plums (*P. domestica* L., *P. salicina* Lindl.), and almond (*P. dulcis* L.) rootstocks, ornamental trees, natural bushes, shrubs are hosts of *Plum pox virus* (PPV). Among them, Japanese flowering cherry (*Prunus serrulata* Lindl. cv. Kanzan) is one of the most popular ornamental trees with light pink double flowers unfolding with 30 petals from crimson buds. It is suggested for landscape architecture and gardening arrangements in all suitable climatic regions because of their mass floral display in the spring season [2]. Previously, PPV was reported on plum, apricot, peach, and cherry trees as well as on some wild prunus species in Europe, Asia, Africa, and North America. Like most stone fruit species, ornamental prunus species are also susceptible to *Plum pox virus* (PPV). For the first time, Sharka disease caused by PPV was described and identified on local plum cultivar trees in Edirne orchards in Türkiye by Sahtiyancı [3]. Later, Yürektürk [4] reported PPV infections on peach, apricot, and plum trees in the orchards of the Marmara region, while Dunez [5] reported the presence of viruses including PPV diseases on stone fruit in the Mediterranean and Near Eastern countries. Besides apricot trees, Elibüyük [6] identified M strain of PPV on ornamental plum trees (*Prunus cerasifera* Pissardii) with its vector *Hyalopterus pruni*. Using aphid transmissions, and bud and chip grafting tests Damsteegt et al. [7] investigated potential host species, reservoir plants, and the host range of PPV in the U.S.A. Screened trees were observed for characteristic symptoms of PPV, and the results were confirmed by applying ELISA and RT-PCR tests. Besides sour cherry (*Prunus cerasus*) and snow fountains (*Prunus snofozam*) 33 prunus species/cultivars were found infected with PPV. By applying ELISA and RT-PCR tests in the Czech Republic, Polak [8] reported *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), *Prunus necrotic ring spot virus* (PNRSV), *Apple chlorotic ringspot virus* (ACLSV), *Apple mosaic virus* (ApMV) and *Cherry leafroll virus* (CLRV) on leaf samples of blackthorn (*Prunus spinosa* L.), plums (*P. domestica* L.) and Prunus *cerasifera*, sour cherry (*P. cerasus*) and sweet cherry (*P. avium*) trees. Tested trees revealed the presence of those viruses at the rate of 74% myrobalan, and blackthorn samples were found infected with PPV, PDV and PNRSV. By employing ELISA and specific PCR for PPV-D

* *Corresponding author: Department of Plant Protection, Faculty of Agriculture, Tekirdağ Namık Kemal University, 59030, Tekirdag, Türkiye*
E-mail address: hilbagi@nku.edu.tr

and PPV-M strains, Sebastian et al. [9] investigated PPV on 120 ornamental prunus species/cultivars, including (*P. domestica* L.), 17 *Prunus serrulata* cultivars in Hungary. As a result of this investigation, 22 species/cultivars were found infected with PPV-D; moreover, two samples were infected with PPV-M and only one sample was found infected with mixed infection of both PPV-D+PPV-M. At least 7 cultivars of *Prunus cerasifera* were infected with PPV-D strain. None of the *P. serrulata* cultivars had any PPV infection at all in Hungary. Using DAS- ELISA and RT-PCR tests, İlbağı et al. [10] observed widespread blackthorn (*Prunus spinosa* L.) bushes all over the hills and pastures in the Trakya region of Türkiye. They investigated for the presence of *Plum pox virus* (PPV), *Apple chlorotic leaf spot virus* (ACLSV), and *Apple mosaic virus* (ApMV) in 54 symptomatic blackthorn leaf samples. Both test results revealed that 13 out of 54 samples at the rate of 24.1% were found infected with PPV. Recently Cıtır et al. [11] identified a new host of PPV on *Tilia* spp. which is often used as valued park trees along avenues and roads because of the aesthetic value in urban landscape practices. Kamenova [12] stated that in Bulgaria, PPV strains on symptomatic myrobalan (*P. cerasifera* Ehrh.) trees. Infected myrobalan trees had a total of 49% PPV, including 57% PPV-M, 29% PPV-D strains and 2% of them had mixed infections of both strains. Only one symptomatic myrobalan was found infected with PPV-Rec strain. Serçe et al. [13] obtained 16 PPV isolates from infected apricot trees in Ankara province of Türkiye. Tested 9 isolates at the rate of 60% were infected with PPV-M and PPV-D and both virus coinfections. The rest of the other PPV isolates, however, revealed different genomic features other than known PPV isolates. Thus, they found a new PPV isolate and suggested the Turkish PPV isolate as symbolized with PPV-T. In order to find out the spreading of PPV strains by aphid vectors in the orchards in Western Türkiye, Çaglayan et al. [14] reported the role of rootstocks in nurseries. They determined that two out of six species of rootstocks, Nemaguard (*P. persica* × *P. davidiana*) and Myrobalan (*P. cerasifera*) were infected with PPV-T more than PPV-M and PPV-D strains. During the investigation, two out of six aphid species, namely *Myzus persicae* and *Hyalopterus pruni*, were found colonized most in especially PPV-T infected rootstocks. İlbağı and Çıtır [15] identified PPV-T strain on almond trees in Trakya. They determined the phylogenetic relationship of PPV-T and other PPV strains between intragroups of almond isolates and other PPV isolates retrieved from different isolates. Gurcan and Ceylan [16] examined 314 out of 612 symptomatic stone fruit leaf samples from different parts of Türkiye by serological and molecular tests and obtained amplifying at 664 nt lengths in P3-6K1 (Nter) CI coding region of PPV genome. Besides, one sample collected from Bursa was found infected with PPV-Rec strain. All the other samples were infected with PPV-D and PPV-T strains collected from the limited number of orchards when comparing PPV-M strain in several orchards and nurseries. Compared to all strains, PPV-T seems to be the dominant strain all over Türkiye. For the determinations of PPV strains in Tekirdağ province, Baş [17] determined five out of 25 *P. ceracifera* Pisasardi Nigra leaf samples infected with PPV by RT-PCR test. However, P. serrulate "Kanzan'', *P. ceracifera* Atropurpurea, and *P. spinosa* L. were found free from PPV.

The aim of this study was to investigate the presence of PPV on Japanese flowering cherry (*Prunus serrulata* Lindl. cv. Kanzan) by using RT-PCR test. In order to determine phylogenetic relationship of the *Prunus serrulate* PPV isolate, the PCR product was sequenced, and partial nucleotide and amino acid sequence of PPV was compared with published sequences of other PPV isolates in the GenBank [18] and EMBL [19] databases.

2. Materials and Methods

Plant sampling: Survey studies were performed in the districts of Çorlu, Malkara, Muratlı and Süleymanpaşa districts in Tekirdağ province in September of 2019. Symptomatic leaf samples from the infected Japanese flowering cherry (*Prunus serrulata* Lindl. cv. Kanzan) trees were collected. Thus, a total of 15 leaf samples such as chlorotic spot symptoms and healthy control leaves were gathered from the survey fields (Figs.1, 2). During surveys it was observed symptomatic and asymptomatic *P. serrulata* trees were side by side on roadsides, streets, parks, and every type of recreational site as well as home gardens of Tekirdağ province in Trakya.

Nucleic acid isolation from PPV-infected samples: Total RNA was extracted from 15 study plant materials using the silica-based method described by Foissac et al. [20].

cDNA synthesis: First strand cDNA was synthesized from total isolated RNA by using RevertAidTM First Strand cDNA Kit (Fermentas; Vilnius, Lithuania). In each reaction, 0.5 µg RNA sample and 20 pmol of reverse complementary primer pair of PPV were used and processed according to the manufacturer's instructions.

RT-PCR amplification: The primer pairs of P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′) and P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′) located at the C-terminus of the PPV CP gene as described by Wetzel et al. [21] were used to amplify a 242 bp product. The PCR reaction for PPV consisted of 2 µl 10x reaction buffer, 3.75 µl MgCl2 (25 mM) 1 µl dNTP (10 mM), 1.5 µl for each primer, 0.2 µl Taq DNA polymerase enzyme (M.B.I. Fermentas) and 10.05 µl RNAse free water. The amplification protocol for PPV was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 62°C for 30 sec, 72°C for 45 sec and the final extension step at 72°C for 10 min in a Techne thermal cycler. PCR products were analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide (EtBr) and viewed under UV Illumination in a gel documentation system (Vilber Lourmat; Marne La Vallee Cedex 1, France).

Sequencing of RT-PCR products: For sequence analysis, PCR products of two isolates found infected with PPV were purified from agarose gels using QIAquick gel extraction kit (M.B.I. Fermentas; St. Leon-Rot, Germany) in accordance with the manufacturer's protocol and were directly sequenced at Refgen (Biotechnology Company, Ankara, Türkiye). Obtained nucleotide and deduced amino acid sequences were aligned with the Bioedit program (version 7.2.5). The alignments were used as input data to construct phylogenetic trees with the neighbor-joining distance method implemented in Mega 7 program [22]. Pairwise sequence comparisons were calculated with the BioEdit program. Bootstrap analysis with 1000

replicates was performed to assess the robustness of the branches.

3. Results and Discussion

In this study, the analysis of the collected leaf samples revealed that *P. serrulata* is a new natural host of PPV in Türkiye. Apart from stone fruit species such as plum, apricot, peach, cherry, and nectarine, wild prunus species also are susceptible to *Plum pox virus* (PPV) as previously reported: the presence of PPV on blackthorn (*Prunus spinosa*) in Trakya region was identified in Tekirdağ by İlbağı et al. [10]. Elibuyuk [6] determined PPV-M strain on ornamental plum trees (*Prunus cerasifera*) with its vector *Hyalopterus pruni* in Ankara. Recently, a new host of PPV in the world was identified on *Tilia* spp. by Cıtır et al. [11]. It has also been reported to infect several ornamental and wild prunus species as natural and experimental hosts in the U.S.A [7]. Moreover, in Bulgaria, Kamenova [12] reported PPV strains on symptomatic myrobalan (*P. cerasifera* Ehrh.) trees. In September 2019, systemic disease symptoms like diffuse chlorotic mosaic and localized spots on the leaves of *P. serrulata* trees as exhibited in Figure 1 and 2 in Tekirdağ were observed. Fifteen leaf samples collected from *P. serrulata* trees on the roadside and in home gardens were tested by RT-PCR in order to determine the presence of PPV. The RT-PCR test results showed that two samples were found infected with PPV. Thus, only two leaf samples enabled the amplification of 242 bp amplicon characteristic of the PPV. The amplicons of two positive isolates amplified by PCR test were sequenced and obtained a partial sequence from one. The partial sequence of PPV was deposited in the GenBank under the accession number MW017468.

The results indicate that Prunus serrulata is Türkiye's new ornamental host of PPV. The present study's results confirm the previous U.S.A studies reported by Damsteegt et al. [7]. They indicated that a wide range of native and ornamental prunus species are susceptible to U.S. isolates of PPV-D. The partial nucleotide sequence of PPV obtained from the P. serrulata isolate was aligned and compared with 22 PPV partial and complete genome sequences retrieved from the GenBank.

Fig. 1 Chlorotic mosaic symptoms on leaves of infected *Prunus serrulata* Kanzan.

Fig. 2 Chlorotic mosaic and leas spots symptoms on *Prunus serrulata* Kanzan.

Sequence analysis revealed that it had 100-44.30% nucleotide and 100-10.97% amino acid identities of PPV isolates depositedin the GenBank from diverse origins around the world. In conclusion, the highest nucleotide and amino acid identities based on CP was 100% with isolates of isAp-87, isAp-117, AnMrPl540, isMrPJ411, and isMrPJ416 from Türkiye while the lowest level of identity was 44.30-10.97% isolate Keşan1 from Tekirdağ, Türkiye. Nevertheless, the highest amino acid identity was 100% with isAp-87, isAp-117, AnMrPl540, isMrPJ411, and isMrPJ416 from Türkiye while the lowest identity was 10.97% with Keşan1 almond isolate from Tekirdağ, Türkiye. Phylogenetic analysis demonstrated that PPV isolates by the neighbor-joining method using MEGA 7 software indicated to be divided into two groups as exhibited in Figure 3. PSer isolate of PPV identified on *P. serrulata* clustered into the first group with twenty PPV isolates. However, Corlu 2 and Kesan 1 almond isolates belonging to PPV-T strain stand out in the second group in phylogenetic tree. PSer isolate of this study clustered other PPV isolated belonging to PPV-M, PPV-D, and PPV-T strains. PPV is a causal agent of Sharka disease on plums which were identified in Edirne province of Trakya by Sahtiyancı [3], and ornamental *Prunus* trees were determined in diverse studies from dissimilar origins worldwide [5, 8, 9, 12]. Up to now, the presence of PPV by previous studies has been identified in stone fruits growing in Türkiye such as peach, plum, apricot, nectarine, and almond [4, 13, 9, 12] as well as on blackthorn, Japanese plum, and rootstock species of Nemaguard and Myrobalan as being the reservoir host of PPV [6, 10, 14]. Moreover, several ornamental and wild *Prunus* species were identified as natural and experimental hosts of PPV in different countries [7]. However, Baş [17] could not identify PPV in six symptomatic *P. serrulata* Kanzan samples collected at the beginning of the summer season on June in Tekirdağ. No information was available on the occurrence as a natural host of PPV on *P. serrulata* Kanzan. To our best knowledge, this is the first identification report of PPV on *P. serrulata* Kanzan in the Tekirdag province of Türkiye. Obtained results would need further studies which include strains of PPV and also phylogenetic relationship of the complete nucleotide sequence of PPV in different geographic locations in the country and worldwide.

Chromosome numbers of the clover genotypes were correlated with their nuclear DNA content by counting mitotic chromosomes of only one white clover genotype with a microscope. Based on cytological investigations, chromosome number of the white clover genotype was determined as $2n = 4x = 32$, indicating that the genotype was tetraploid (Fig 3). The rest of the white clover genotypes were assumed to be tetraploid with $2n = 4x = 32$ chromosomes since they had similar 2C nuclear DNA content values. The results of cytological investigations obtained in the current study are in agreement with the known chromosome number of white clover [30, 39].

4. Conclusion

Plum pox virus (PPV) is the causal agent of Sharka disease which is one of the most devastating viral pathogens in stone fruits. The virus infects stone fruit species such as peach, nectarine, plum, cherry, and

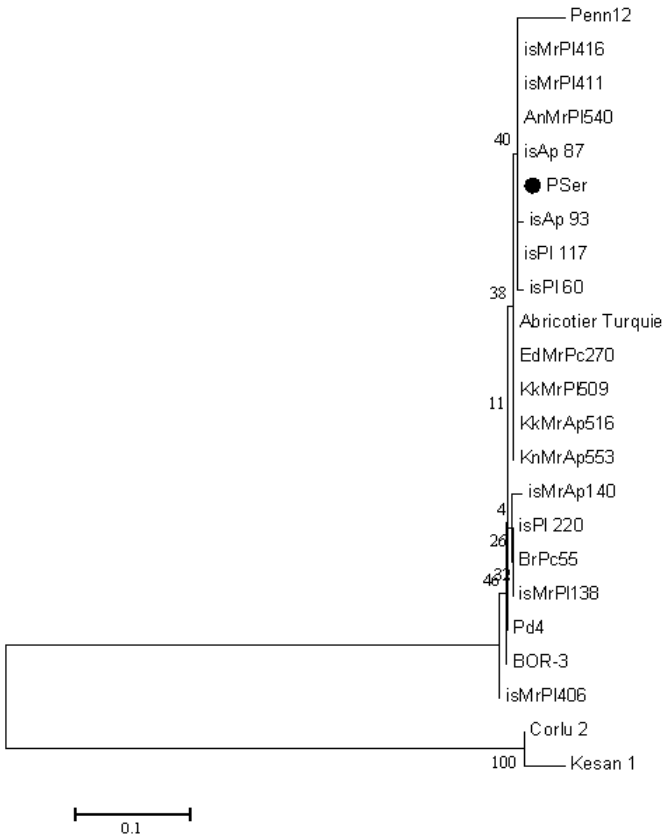

Fig. 3 Constructed phylogenetic tree based on partial nucleotide sequencing of *Plum pox virus* (PPV) on *Prunus serrulata* Kanzan.

apricot, resulting in extensive economic losses as well as on ornamental plants and wild natural plants. PPV is also a viral pathogen in quarantine pathogen lists which might be transported long distances through infected plant materials internationally, and is transmitted from the infected tree by insect vectors or grafting. In Türkiye, so far, PPV has been identified on stone fruits like plum, nectarine, apricot, peach, ornamental, and wild nature plants. However, no information is available about the occurrence as a natural host of PPV on *Prunus serrulata* Kanzan. In this study, 15 leaf samples collected from *P. serrulata* trees were screened by RT-PCR test to identify the presence of PPV. Two out of the fifteen samples were found infected with PPV. The PCR products of two isolates were sequenced. Sequence analysis showed that the nucleotide and amino acid identities were between 100-44.30% and 100-10.97% with other PPV isolated retrieved from the GenBank, respectively. To our best knowledge, this is the first report of PPV on *P. serrulata* Kanzan in Türkiye.

Declaration

Author Contribution: Conceive-H.I.; Design-, H.I.; Supervision- H.I.; A.Ç., Experimental Performance, Data Collection and/or Processing H.I.; B.B., Analysis and/or Interpretation H.I., Literature Review- H.I.; A.Ç., Writer-H.I.; A.Ç, Critical Reviews - H.I.; A.Ç.

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Orcid-ID

Havva ILBAGI https://orcid.org/0000-0002-0016-190X
Busra Bas https://orcid.org/0000-0003-2136-2361
Ahmet ÇITIR https://orcid.org/0000-0002-0453-5112

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