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Identification and genetic characterization of *Pseudomonas syringae* pv. *syringae* from sweet cherry in Turkey

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Abstract

Pseudomonas syringae pv. *syringae* (*Pss*), which causes bacterial canker, is the most polyphagous bacterium in the *P. syringae* complex due to its broad host range. This pathogen is considered the major bacterial disease in cherry orchards. In this study, several samples were collected from infected sweet cherry trees in different locations of the Marmara region in Turkey between 2016-2018. Sixty-three isolates were identified as *Pss* by pathogenicity, LOPAT, GATTa, and MALDI-TOF MS tests. Total genomic DNA was extracted to confirm identity, followed by PCR amplification of *syrB* and *cfl* genes. Out of 63 isolates, 12 were randomly selected for Repetitive Element Sequence-based PCR (rep-PCR) and Multilocus Sequence Typing (MLST) analysis to gain insight into the relationships of those isolates. The cluster analysis of rep-PCR (ERIC-, REP- and BOX-PCR) could classify the isolates into two distinct clusters. Phylogenetic analysis was carried out to obtain the relation between isolates and the location.The MLST analysis of *gyrB*, *rpoDp, rpoDs*, and *gltA* genes allowed a clear allocation of the isolates into two separate main clusters. The relationship among the isolates were also evaluated by constructing a genealogical median-joining network (MJN). The isolates from six locations produced 11 haplotypes that were illustrated in the MJN. The results of this study proved that location could not be an indicator for showing the genetic diversity of *Pss* from cherry orchards. As the genetic variability of *Pseudomonads* has been demonstrated, the current study also showed high diversity among different isolates even within the populations. While more research is recommended, the results of this study contributed to a better understanding of the *Pss* evolutionary progress and genetic diversity of sweet cherry isolates.

Keywords: Bacterial canker, *Prunus avium*, Repetitive Element Sequence-based PCR, Multilocus Sequence Typing, phylogenetic analysis

Sweet cherry (*Prunus avium* L.) is a fast growing deciduous tree in the *Rosaceae* family. It requires mild temperature and mild humidity during the growth period. High or low temperatures may have a negative effect on cherry production and fruit quality (Burak 2003). Sweet cherry ripens first among stone fruits, followed by apricot, peach, and plum. Since sweet cherry is the first on the fresh market, it is in high demand in late spring and early summer (Bujdoso and Hrotko 2007). Global cherry production was over 2.5 million tons in 2019 (FAO 2019) and major cherry-producing countries include Turkey, the United States, Chile, Uzbekistan, Iran, Spain, and Italy. Of the total world production, approximately 42% originated from Turkey and Europe and about 14% from the United States. Sweet cherry is considered one of the most economically important fruit products in Turkey (FAO 2019). Generally, the increase of cherry production and quality is related to climate, environmental factors, and pollination. Most sweet cherry varieties including 0900 Ziraat, which is an economically important and common variety in Turkey, are self-incompatible and require cross-pollination with another as pollen sources like Lambert, Merton Late, Starks Gold, or Stella. So, it is essential to use pollination cultivar to increase yield in cherry cultivation (Özçağıran et al. 2005).

Diseases, such as fungal, bacterial, and viral, can affect cherry production. Bacterial canker, caused by *Pseudomonas syringae* pv. *syringae* (*Pss*) is one of the major diseases associated with sweet cherry production and causes significant losses (Kennelly et al. 2007). The pathogen became more prevalent over the past decades, especially in young plantations of stone fruits with intensive production systems (Vicente et al. 2004; Sulikowska and Sobiczewski 2008; Gilbert et al. 2009; Spotts et al. 2010). The *P. syringae* pathovar contains pathogenic members in over 180 plant species, including stone fruits such as sweet cherry (*P. avium*), sour cherry (*P. cerasus* L.), apricot (*P. armenica* L.), peach (*P. persica* L.), and plum (*P. domestica* L.)

(Braudbury 1960). The disease symptoms include blossom blast and spur dieback, leaf and fruit lesions, cankers associated with gummosis of woody tissue, loss of scaffold limbs, and overall decreased fruit yields (Crosse 1966; Jones 1971; Kennelly et al. 2007). The disease cycle of *Pss* is initiated in spring by colonization and development on blossoms. Blast symptoms can occur following extended periods of cold and wet weather or after a frost event (Chandler and Daniell 1976; Kennelly et al. 2007; Spotts et al. 2010). The pathogen can result economic lossess due to reduction in fruit and death of branches or trees (Bulbul and Mirik 2014). Bacterial canker inccidence was reported as approximately 30% on apricot and 5% on plum in Mediterranean region of Turkey and as 58.9% on cherry in Marmara region of Turkey (Cetinkaya-Yildiz et al. 2016; Oksel and Mirik 2021).

Pathovars of the *P. syringae* complex have traditionally been characterized biochemically and described by several tests such as LOPAT (L-levan production; O-oxidase production; P-pectinolytic activity; A-arginine dihydrolase production and T-tobacco hypersensitivity) and GATTa (G-gelatin liquefaction; A-aesculin hydrolysis; T-tyrosinase activity; Ta-utilization of tartrate) (Lelliott et al. 1966; Latorre and Jones 1979; Schaad et al. 2001). They have also been identified by toxins, which are important virulence factors (Mitchell 1991). The *Pss* isolates were found to produce syringomycin as a toxin. Furthermore, it could be detected using PCR using specific primers to amplify the *syrB* gene encoding these metabolites (Sorenson et al. 1998). Although these tests are effective, bacterial phenotypical characterization alone often fails to reveal genetic relationships within or between species.

DNA fingerprinting has proved to be functional for analyzing genotypic distribution and estimation of genetic relatedness of different organisms, including bacteria. Using DNA fingerprints, the *P. syringae* has been divided into at least 60 pathovars based on their hosts. The

DNA homology analysis has grouped them into nine genomospecies (Garden et al. 1999; Young 2010). Specific genomic fingerprints have been proposed as diagnostic tools using amplification of interspersed repetitive DNA sequences (Versalovic et al. 1994; Rademaker and Bruijn 1997). These DNA sequences are presented in bacterial genomes referred to rep-PCR (Repetitive element sequence-based PCR fingerprinting) using primer sets of ERIC (Enterobacterial Repetitive Intergenic Consensus), REP (Repetitive Extragenic Palindromic), and BOX (BOX-A1R-based Repetitive extragenicpalindromic) (Rademaker and Bruijn 1997). The DNA fingerprinting studies had shown that the *P. syringae* isolates were distributed among different groups and did not classify together even when isolates were isolated from the same species (Stead et al. 2003; Kaluzna et al. 2010). Thus, the lack of isolate relatedness with host and species is a common theme in the overall analysis of *P. syringae*.

Various other molecular techniques have been used to characterize *Pss* isolates. They demonstrated that a high genetic heterogeneity existed in the isolates (Little et al. 1998; Scortichini et al. 2003; Cirvilleri et al. 2006; Dillon et al. 2021). These observations have been confirmed in another powerful DNA fingerprinting method called Multilocus Sequencing Typing (MLST) (Sarkar and Guttman 2004). The MLST studies provide fundamental insights into their phylogenetic and taxonomic relationships (Sarkar and Guttman 2004; Bull et al. 2011; Butler et al. 2013). The *P. syringae* species have been categorized into 13 phylogroups by MLST (Berge et al. 2014; Baltrus et al. 2017). The recent studies show that it is possible to carry out an average nucleotide identity analysis of conserved and shared genes between two bacterial isolates. It has been proposed based on pairwise genome comparisons as new standards for the definition of prokaryotic species (Konstantinidis and Tiedje 2005; Boyer et al. 2009; Sawada and Fujikawa 2019).

This study aimed to identify and differentiate 63 *P. syringae* isolates from diseased sweet cherry using pathogenicity, biochemical, and molecular analysis. The rep-PCR, including ERIC, REP, BOX, as well as MLST analysis were used to classify and discriminate the *Pss* isolates from diseased sweet cherry from different locations of the Marmara region, Turkey.

Materials and Methods

Plant material and sampling. Survey studies were conducted in different cities including Balıkesir, Bursa, Canakkale, Edirne, Kocaeli, Kırklareli, Istanbul, Tekirdag, and Yalova of the Marmara region in Turkey during the growing seasons of 2016 to 2018. Samples were collected for the presence of symptoms referring to possible bacterial diseases from *Prunus avium* cv. 0900 Ziraat. In addition, symptomatic plant materials (leaf spot, branch necrosis, blossom blast or necrotic buds) were collected from March until the end of September (Supplementary table 1).

Isolation and purification of bacterial isolates. Small fragments were taken from the border area between apparently healthy and diseased tissue, shortly disinfected with 70% ethanol. Then, the pieces were macerated in sterile saline buffer (2% NaCl). Serial dilutions of the resulting tissue extract were plated onto Pseudomonas F Agar (PSF) medium and incubated at 27°C for 48 h (King et al. 1954). Next, single colonies, morphologically resembling *P. syringae* were picked and re-streaked on the same medium to ensure purity (Lelliott and Stead 1987).

Pathogenicity test. The pathogenicity tests were conducted using *Prunus avium* cv. 0900 Ziraat. The pathogenicity of isolates was tested in laboratory conditions using detached immature

cherry fruits (Moragrega et al. 2003) and two-years-old young cherry trees in 48x40 cm diameter black plastic pots filled with soilless potting mix (Vicente et al. 2004; Hulin et al. 2018). Fruits were sterilized in 0.5% hypochlorite for 5 minutes and rinsed with sterile distilled water (SDW). Bacteria were scraped from 2-day-old cultures on PSF plates. Bacterial suspension of 10^8 cfu/ml (OD_{600}:0.2) were prepared and injected into fruits placed in transparent boxes that included moist tissue paper to maintain high humidity, and incubated at 22°C (16 h light, 8 h dark). The experiment was assesed after two weeks. Pathogenicity test using young cherry tree was conducted on the isolates that appeared virulent on cherry fruits with black necrotic lesions. Bacterial suspensions were prepared the concentration of 10^8 cfu/ml (OD_{600}:0.2). A sterile scalpel was used to wound the branches of young cherry trees . After that 200 µl of bacterial suspension was pipetted into the wound and the inoculation sites were covered with parafilm for 24 h. The young cherry trees were kept in climatic room at 25±2°C with 16 h photoperiod. The experiment was assesed after two months. SDW and pathovar reference *Pss* Naip1 isolate were used as negative and positive controls for both detached immature cherry fruits and young cherry plant pathogenicity tests, respectively. Both assays were performed three times with three replicates for each isolate. Symptom development was observed and compared with controls after inoculation.

Phenotypic characterization. Bacterial isolates and reference isolate (*Pss* Naip1) were tested according to Gram reaction and LOPAT (Levan production, Oxidase reaction, Potato soft rot, Arginine dihydrolase activity, and Tobacco hypersensitivity) character. Also, GATTa test (Gelatine liquefaction, Aesculine hydrolysis, formation of Tyrosinase and Tartrate metabolism) was performed to differentiate the species of *P. syringae* (Lelliottt and Stead 1987; Kaluzna et al. 2012). All tests were performed three times with three replicates for each isolate.

Identification of bacterial isolates with MALDI-TOF mass spectrometry. These analyses were conducted in the Center for Implementation and Research of Plant Health Clinic at Mustafa Kemal University, Hatay, Turkey. To yield colonies, bacterial isolates were subcultured on PSF medium plates and grown at 27°C for 48 h. After that, the colonies were scraped with a sterile scalpel. The method that has been described for the preparation of bacteria for identification by MALDI-TOF MS is the standard ethanol-formic acid protein extraction in 70% formic acid. One µl of the mixture was directly spotted onto a polished steel target plate (Bruker Daltonics, Bremen, Germany). Measurements were performed using Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany). Each bacterial isolate was measured using mass spectra compared with the BioTyper database that is a part of MALDI BioTyper 2.0 software (Bruker Daltonics, Bremen, Germany). It contained reference spectra of a broad number of microbial species. For bacterial isolates identification, the raw spectra of the unknown bacteria were used for pattern matching compared to the reference spectra of the database. The results of the pattern-matching process were expressed as proposed by the manufacturer, with log (score) values ranging from 0 (no similarity) to 3 (absolute identity) (Carolis et al. 2012; Ziegler et al. 2012; Uysal et al. 2019).

DNA extraction and PCR amplification. Bacterial isolates were cultured on PSF medium and incubated for 48 h. Total genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) directly from the cultures. DNA concentration and purity were verified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Amplification was performed in a total volume of 25 µl. The reaction mixture consisted of 5.5 µl nuclease-free water, 12.5 µl 2X master mix (GoTaq PCR kit, Promega, Madison, WI, USA), 2.5 µl of each primer (10 µM), and 2 µl (5 ng/ µl) of DNA.

Amplification was carried out using a thermal cycler (Biorad Laboratories, Inc., Hercules, CA, USA).

Detection of genes coding syringomycin and coronatine. Determination of genes coding synthesis of the bacterial phytotoxins syringomycin and coronatine was carried out by PCR with *syrB* encoding synthesis of syringomycin with primers syrB1/syrB2, and *cfl* encoding coronatine synthesis with primers CFLF/CFLR. The isolates expected to produce band sizes of 752 bp and 650 bp in *syrB* and *cfl*, respectively (Bereswill et al. 1994; Abbasi et al. 2013). The PCR reaction was performed in a Thermo Cycler TC-412 (Techne, Keison Products, Chelmsford, UK). Reference *Pss* Naip1 was included in all reactions. PCR products were separated in a 1% agarose gel in 0.5XTBE buffer by comparison with 100 bp DBA ladder (ThermoScientific, SM0623, Vilnius, Lithuenia), and gel electrophoresis was run for 1 h at 5 V/cm. After staining in ethidium bromide (0.5 mgml -1), the gel was washed with distilled water and visualized under UV light (Bio-Rad Labotories, Hercules, CA, USA) (Sambroak et al. 1989). Twelve confirmed *Pss* isolates from the sweet cherry in different locations of Marmara Region, Turkey, were randomly selected for rep-PCR and MLST analysis (Table 1).

rep-PCR. The rep-PCR was performed using the ERIC, REP, and BOX primers (Louws et al. 1994) (Table 2). The PCR programs were comprised: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 44°C (REP), 52°C (ERIC) and, 53°C (BOX) for 1 min, extension at 72°C for 1 min, and a single final extension cycle at 72°C for 5 min. PCR amplifications were carried out three times with all DNA samples. The PCR products were separated by gel electrophoresis on 1.5% agarose gel in 0.5XTBE buffer for 7.30 h at 40 V. After electrophoresis, the gel was then kept in the ethidium bromide (0.5 mg l^{-1})

for 20 min and transferred into distilled water for 20 min. Patterns were visualized under UV (Gel Doc™ EZ Imager, Bio-Rad Labotories, Hercules, CA, USA) and analyzed.

MLST. The MLST was carried out using genes and primer sets listed in Table 2. The protocols described by Sarkar and Guttman (2004) and Hwang et al. (2005) were used. The housekeeping genes of *rpoDs* and *rpoDp* based on *rpoD* encoding sigma factor 70, *gyrB* encoding DNA gyrase B, and *gltA* encoding citrate synthase were used. The PCR amplifications were as follows: initial denaturation at 94°C for 3 min, 32 cycles of denaturation at 94°C for 30 sec, annealing at 63°C, 63°C, 63°C, and 64°C for 30 sec for *gyrB, rpoDp, rpoDs*, and *gltA*, respectively, extension at 72°C for 30 sec, and final extension at 72°C for 5 min. The PCR products were purified using the PCR purification kit (Wizard® SV Gel and PCR Clean-up kit, Promega, Madison, WI, USA) and the sequencing was carried out by GenHunter Sequencing Service in Nashville, TN, USA.

Phylogenetic and statistical analyses. The sequences were revised by ChromasPro version 1.7.4 (http://technelysium.com.au/wp/) and aligned using MEGA6 software (Pennsylvania State University, University Park, PA, USA) (Tamura et al. 2013). The consensus sequences were deposited into the National Center for Biotechnology Information (NCBI) GenBank database (Table 3). In order to evaluate the taxonomic relation between *Pss* isolates obtained from the sweet cherry grown in different locations, phylogenetic analysis was performed. The neighbor-joining (NJ) method was used to construct a phylogenetic tree of MLST housekeeping genes. Bootstrap phylogeny analysis was done with 1,000 replications to be able to test the trees statistically. The pairwise genetic distance was calculated by the Jukes-Cantor method using MEGA6 software (Tamura et al. 2013). DNAsp version 5.1 software (University of Barcelona, Barcelona, Spain) was used to compute haplotype data file (Librado

and Rozas 2009). To estimate the significance of variance within and among the isolates from different locations, the AMOVA (Analysis of Molecular Variance) was performed by Arlequin version 3.5 software (Excoffier and Lischer 2010). The network version 4.6 software was used to obtain genealogical differences between isolates from different locations (Bandelt et al. 1999).

Results

Isolation and purification of bacterial isolates. During the 2016-2018 survey of sweet cherry orchards in the Marmara region of Turkey, 152 plant samples with symptoms referring to possible bacterial disease were collected. Even though samples of diseased sweet cherries were from nine different cities in the Marmara region of Turkey, the isolates were obtained in six out of nine cities. There were no isolates obtained from Kocaeli, Yalova, and Edirne.

After isolation, the bacterial colonies that were white and fluorescent on PSF medium were selected and purified. As a result, a total of 82 bacterial isolates were obtained from sweet cherry orchards in Balıkesir, Bursa, Canakkale, Kırklareli, Istanbul, and Tekirdag. All isolates were obtained from sweet cherry cv. 0900 Ziraat. We did not obtain any isolates from other cultivars that were used for pollination such as Lambert and Klasik.

Pathogenicity test. Sixty-three isolates out of eighty-two bacterial isolates produced black necrotic lesions on sweet cherry fruits 7-10 days after inoculation. The isolates that produced black necrotic lesions on cherry fruits were selected to be used for pathogenicity test on young cherry trees. Twelve aggressive isolates showed brown/black lesions into the branches of young trees after two months of inoculation. Reference *Pss* Naip1, used as a positive control in the pathogenicity test, also caused the same symptoms on immature cherry fruits and young cherry

trees. The bacterium was re-isolated from necrotic lesions as described previously. All negative control fruits remained symptom- free and the bacterium was not isolated from fruit and young cherry tree tissues.

Phenotypic characterization. Sixty-three isolates were Gram negative and produced fluorescent pigment on PSF medium. All isolates produced levan on Sucrose Nutrient Agar (SNA) medium also produced water-soaked and necrotic lesions on tobacco leaves. Therefore, those characters of sixty-three isolates have revealed that they belong to *Pseudomonas* group 1a. The results of GATTa tests showed the ability to gelatine liquefaction and aesculin hydrolysis of the sixty-three isolates. Therefore, all sixty-three isolates were identified as *Pss*.

Identification of bacterial isolates with MALDI-TOF mass spectrometry. In parallel with the pathogenicity, LOPAT, and GATTa tests, the isolates were analyzed by MALDI-TOF MS. The log (score) values of the twelve selected isolates from different locations were higher than 2.0. Therefore the twelve isolates were identified as *Pss.*

Detection of genes coding for syringomycin and coronatine. The syringomycin-specific product of 752 bp of the *syrB* gene was detected in sixty-three isolates. However, the expected size (650 bp) product obtained by amplification of *cfl* gene encoding for coronatine was not obtained for any isolates. Based on these results, the isolates from sweet cherry were identified as *Pss*.

rep-PCR. The fingerprint patterns of rep-PCR using ERIC, REP, and BOX primers were shown in Figure 1. The patterns were very different and complex. The rep-PCR yielded distinct products, ranging from 150 bp to 4.5 kb, and 63 bands were scored. Differences among isolates were visually evaluated based on the product weight and migration status of amplicons.

The REP-, BOX-, and ERIC-PCR yielded 15, 14, and 12 polymorphic patterns, respectively. Thus, they were very efficient for analyzing the genetic diversity of the isolates used in this study. The BOX-PCR alone proved polymorphic enough that it could generate similar genetic differentiation as the combined primers patterns (Figs. 1 and 2). The combined dendrogram of REP, ERIC, and BOX-PCR grouped the isolates into seven clusters (Fig. 2). The first cluster included *Pss* isolates (C109, C110, and C112) from three regions of Kırklareli, Tekirdag/Yeniciftlik, and Bursa/Osmangazi. Cluster 2 comprised one isolate (C103) from Canakkale/Lapseki region. Cluster 3 grouped the isolates (C101 and C102) from Tekirdag/Naip and Balıkesir regions. The fourth cluster included one isolate (C113) from Tekirdag/Kirazlı region. Cluster 5 comprised an isolate (C104) from Canakkale/Lapseki. Cluster 6 grouped two isolates (C105 and C106) from Canakkale/Bayramic and Canakkale/Lapseki regions. The last cluster included two isolates (C107 and C108) from Tekirdag/Marmaraereglisi and İstanbul/Selimpasa regions (Fig. 2).

MLST. In the current study, MLST was performed based on three housekeeping genes of *gyrB*, *rpoD*, and *gltA* (Sarkar and Guttman, 2004). To establish the phylogenetic position of the *Pss* isolates from this study, a neighbor-joining phylogenetic tree was constructed with the concatenated sequences of the *gyrB*, *rpoD* (*rpoDs* and *rpoDp*), and gltA genes (Fig. 3). The obtained sequences were submitted into the NCBI GenBank database through the BankIt submission tool. The GenBank accession numbers were listed in Table 3.

The tree showed two related phylogenetic groups, one with seven isolates including C101, C102, C103, C105, C107, C109, and C113 originated from Tekirdag/Naip, Balikesir, Canakkale/Lapseki, Canakkale/Bayramic, Tekirdag/Marmaraereglisi, Kirklareli, and Tekirdag/ Kirazli regions. The second group consisted of the remaining isolates, including C104, C106,

C108, C110, and C112 originated from Canakkale/Lapseki, Istanbul/Selimpasa, Tekirdag/ Yeniciftlik, and Bursa/Osmangazi regions. The C112 isolate was phylogenetically more distinct, but it showed to be more related to the second group of isolates (C104, C106, C108, and C110) (Fig. 3).

Phylogenetic Analysis. Several nucleotide data parameters of the *gyrB*, *rpoDs*, *rpoDp*, and *gltA* genes as well as the concatenated sequences of them, were shown in Table 4. The total conserved site was obtained 96.81% for concatenated *gyrB, rpoDs, rpoDp,* and *gltA*. The *rpoDp* showed the highest percentage of variation among the individual genes and the lowest conserved sites. Moreover, the parsimony-informative sites were the lowest, and singleton sites were the highest for this gene. The *gltA* gene was only the one that showed zero singleton site, but the overall mean distance (diversity) was the greatest. Thus, the concatenated sequences demonstrated a considerable variation for the genetic classification of the isolates used in this study.

The genetic variation among and within the locations was also estimated by the AMOVA using the concatenated sequences. The results indicated that the percentage of variation within the locations (74.70) is far greater than among the locations (25.30). The AMOVA and proportion of genetic variation were computed to test genetic structure of the isolates, and to test if there is any association among and within the locations. The relationships of *Pss* isolates from different locations were evaluated by constructing the genealogical median-joining network (Fig. 4). The concatenated sequences of genes gave rise to 11 unique haplotypes that belonged to six different locations. According to the network tree, haplotype 9 (Istanbul) was segregated from the other isolates. This haplotype was connected to other haplotypes by several median vectors and mutations. The haplotypes 5, 6, 7, and 8 belonged to the same region (Canakkale), but they

demonstrated genetic variation among them. They were connected by several median vectors and mutations. Similarly, haplotypes 1, 2, and 3 were from the same location (Tekirdag), but they showed to be genetically divergent. The haplotype 11 from the Bursa region was separated from other species by the presence of mutations and median vectors. Similarly, the haplotype 10 from Kirklareli separated by mutations and median vectors. Lastly, the haplotype 4 (from Balikesir) was separated from haplotype 1, which is from Tekirdag region, but they revealed a close relationship.

Discussion

Pseudomonas syringae pv. *syringae* (*Pss*) isolates are the most polyphagous bacteria in the *P. syringae* complex, based on their wide host range (Kennelly et al. 2007). Therefore, due to the great spread capacity on different hosts, it is essential to determine the genetic heterogeneity of *Pss* isolates originating from various sources (host/location) (Ivanovic et al. 2012; Ilicic et al. 2016). In this study, *Pss* isolates were obtained from the sweet cherry in the Marmara region of Turkey.

Isolates of *Pss* have been characterized conventionally by several assays, including pathogenicity and biochemical tests (Mohammadi et al. 2001; Scholz-Schroeder et al. 2001; Vicente et al. 2004; Bultreys and Kaluzna 2010; Gasic et al. 2012; Ilicic et al. 2016; Runielli et al. 2019; Uysal et al. 2019). The disease symptoms were reproduced on the inoculated immature cherry fruits and young cherry trees using the pathogenicity test. The use of detached fruits was promising and provided reliable results. Other studies also verified that the symptoms develop quickly and clearly on the immature cherry fruits (Moragrega et al. 2003; Bedford et al. 2003).

The LOPAT test has been used to differentiate *P. syringae* from other fluorescent *Pseudomonas* species (Lelliott et al. 1966). All of the sixty-three isolates formed levan type-colonies, and the hypersensitive reaction of tobacco leaves showed a water-soaked and necrotic appearance. The *P. syringae* pathovars affecting stone fruits can be differentiated using another test known as GATTa (Popovic et al. 2021). All of the sixty-three isolates matched with the control *Pss* Naip1. The isolates showed the ability to liquefaction of gelatine and hydrolysis of aesculin. As a result, they showed positive results similar to the previous studies (Kaluzna et al. 2012; Gasic et al. 2012). The pathovars of *P. syringae*, associated with stone fruits and nuts, produce several well-characterized phytotoxic compounds used for pathovar differentiation. Syringomycin is one of the phytotoxic compounds use to identify *Pss* (Sorenson et al. 1998; Scortichini et al. 2003; Gilbert et al. 2009). In the current study, the *syrB* gene coding for syringomycin synthesis was detected in 63 tested isolates. The twelve randomly selected isolates in this study were also identified as *Pss* by MALDI-TOF MS (Uysal et al. 2019). The previous studies have consistently demonstrated the ability of MALDI-TOF MS to describe antagonist bacterial isolates from some fruit orchards for several bacteria, e.g. *Bacillus endophyticus, B. cereus, B. mojavensis, B. megaterium, B. pumilus, B. subtilis, B. amyloliquefaciens, B. vallismortis*, and *Erwinia herbicola* from pomegranate and also *Pseudomonas* sp., *Serretia* sp., and *Pantoea* sp. from apricot (Kara et al. 2019; Atay et al. 2019). However, to our knowledge, this is the first identification of *Pss* isolates from sweet cherry in Turkey using a MALDI-TOF MS test.

Several molecular techniques have been proposed to study the genetic diversity of *Pss* isolates (Natalini et al. 2006; Ivanovic et al. 2012). Among the methods, rep-PCR and MLST have proven promising for the phylogenetical relations of *P. syringae* isolates (Vicente and Roberts 2007; Martin-Sanz et al. 2013). The rep-PCR method has been useful in figuring out the

similarity among *Pss* isolates (Kaluzna et al. 2010; Ivonovic et al. 2012; Gavrilovic et al. 2013). In this study, the rep-PCR using the combined REP-, ERIC-, and BOX primers were used. The constructed dendrogram showed considerable divergence among the isolates (Fig. 2). However, a specific pattern to relate this divergence to the isolation location was not found. Ilıcıc et al. (2016) used rep-PCR to examine the *Pss* isolates from the sweet cherry of different origins but could not exhibit significant differences and patterns among them. They reported that the rep-PCR could be an advantageous technique for determining the genetic diversity of *Pss* isolates. *P. syringae* isolates have been the most commonly used ones for identifying, classifying, and analyzing the various pathogens that cause stone fruit bacterial cankers. In particular, REP-PCR analysis is a highly discriminative method adapted for genetically characterizing bacterial isolates between and within *P. syringae* isolates (Gilbert et al. 2009), including *Pss* (Scortichini et al. 2003) as being similar to our results.

MLST analysis based on three housekeeping genes of *gyrB, rpoD*, and *gltA* could discriminate the *Pss* isolates used in the current study (Fig. 3). The earlier MLST studies have provided fundamental information on their phylogenetic and taxonomic relationships (Bull et al. 2011; Butler et al. 2013). In a study, Yamamoto et al. (2000) analyzed the sequence alignments of two housekeeping genes (*rpoD* and *gyrB*) and demonstrated great effectiveness of these markers in differentiating at the intraspecies level. According to the results from the current study, two main clusters were distinguished with bootstrap value. The isolates were classified under these two main clusters, but they could be distinguished individually.

Generally, the concatenated sequences of the genes have shown high variability. Kaluzna et al. (2010) confirmed that MLST affords the highest discrimination among the *Pss* isolates from hazelnut and stone fruits. They indicated that there was no variation observed from the

place and year of isolation. In the current study, the results from the constructed phylogenetic tree and neighbor-joining network showed that the isolates were not distinguished based on the location from which they were isolated. For example, the isolates C105 and C106 were isolated from the same region but they did not classify together. The AMOVA results also indicated that the percentage of variation within the locations (74.70) is far greater than among the locations (25.30). Vicente et al. (2004) reported a highly heterogeneous *Pss* population isolated from sweet cherry and wild cherry. In the other studies, high genetic differences among isolates originating from diverse *Prunus* species were reported (Gilbert et al. 2009; Abbasi et al. 2013). The *Pss* isolates have been revealed to be the most heterogeneous and intermingled to host plant, year, and place of isolation.

The network analysis can deliver a better understanding of the relationships of the isolates from different locations (Fig. 4). The network tree showed that the *Pss* isolates from the same or different locations could be categorized together or independently. That may attribute to the spread of the isolates from a limited geographic area, adapting to a specific ecological niche, isolation from one host plant, or the adaptation to its environment at the time (Denny et al. 1988; Little et al. 1998; Sisto et al. 2007; Baltrus et al. 2017). As far as is known, the *P. syringae* species complex is divided into 13 phylogroups groups based on MLST (Berge et al. 2014). According to Sarkar and Guttman (2004), the core genome of all *P. syringae* is responsible for determining host specificity. Therefore, it can be assumed that the genetic variation in the housekeeping genes would be very tightly associated with the isolation host. However, there have been a few cases where isolates isolated from the same host turn out to be highly divergent (Vicente and Robert 2007; Kaluzna et al. 2010; Abbasi et al. 2013). It can be associated with a

wide range of virulence factors such as type III secreted effector proteins, toxins, and resistance genes (Sarkar and Guttman 2004).

The results showed that the representatives of *Pss* isolates from different locations can be placed in the same cluster and vice versa. As it is known, *P. syringae* is a common environmental and complex bacterium. Therefore, the pathogen can be adapted easily in different conditions. The pathogenicity, biochemical tests, MALDI-TOF MS, and PCR provided very useful information to correctly identify the isolates of *Pss*. According to the results, one test is not enough to identify bacterial isolates. Therefore more than one identification test should be used. The virulence factor mechanism or adaptation to a specific ecological niche or environment can be a reason for such a variation (Little et al. 1998; Scortichini et al. 2003; Sarkar and Guttman 2004; Kaluzna et al. 2010; Lo et al. 2017). The results from the current study can contribute to a better understanding of the genetic structure, evolution, and genetic diversity of *Pss* isolates from sweet cherry. The results may be helpful to improve further studies on stone fruits.

Acknowledgments

This work was supported by the Scientific and Technological Research Council of Turkey under award number TUBITAK 2214-A 1059B141801417 from and the National Institute of Food and Agriculture (NIFA), United States Department of Agriculture (USDA) Evans-Allen grant, under award numbers TENX-1926-CCOCP and TENX-S-1083. We also would like to acknowledge the support of the Otis L. Floyd Nursery Research Center, Tennessee State University, and the Center for Implementation and Research of Plant Health Clinic at Hatay Mustafa Kemal University.

References

Abbasi, V., Rahminian, H. and Ghanbari, M.A.T. 2013. Genetic variability of Iranian strains of *Pseudomonas syringae* causing bacreial canker disease on stone fruits. Eur J Plant Pathol, 135: 225-235.

Atay, M., Kara, M., Uysal, A., Soylu, S., Kurt, Ş. and Soylu, E.M. 2019. *In vitro* antifungal activities of endophytic bacterial isolates against postharvest heart rot disease agent *Alternaria alternata* in pomegranate fruits. Acta Hortic., 1289:309-314.

Baltrus, D.A., McCann, H.C. and Guttman, D.S. 2017. Evolution, genomics and epidemiology of *Pseudomonas syringae*: challenges in bacterial molecular plant pathology. Molecular Plant Pathology, 18: 152– 168.

Bandelt, H.J., Forster, P. and Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol, 16(1): 37-48.

Bedford, K.E, Sholberg, P.L. and Kappel, F. 2003. Use of a detached leaf bioassay for screening sweet cherry cultivars for bacterial canker resistance. Acta Hortic., 622:365–368.

Bereswill, S., Bugert, P., Völksch, B., Ullrich, M., Bender, C.L. and Geider, K. 1994. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of amplification products. Applied and Environmental Microbiology: 2924-2930.

Berge, O., Monteil, C.L. and Bartoli, C. 2014. A user's guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex. PLoS One 9:e105547

Boyer, K. K., Prudhomme, A. M. and Chung, W. 2009. The last mile challende: evaluating the effects of customer density and delivery window patterns. Journal of Business Logistics, 30(1): 185-201.

Bujdoso, G. and Hrotko, K. 2007. Performance of three sweet cherry and one sour cherry cultivars on dwarfing rootstocks in central Hungary. Acta Horticulturate, 935: 153-160.

Bull, C.T., Clarke, C.R., Cai, R., Vinatzer, B.A., Jardini, T.M. and Koike, S.T, 2011. Multilocus sequence typing of *Pseudomonas syringae sensu lato* confirms previously described genomospecies and permits rapid identification of *P. syringae* pv. *coriandricola* and *P. syringae* pv. *apii* causing bacterial leaf spot on parsley. Phytopathology, 101: 847– 58.

Burak, M. 2003. Meyvecilik-1: 147-151.

Bulbul, M. And Mirik, M. 2014. Prevalence, isolation and identification of bacterial canker pathogens on sweet cherry trees in Tekirdag. The Journal of Turkish Phytopathology, 43:15-29.

Bultreys, A. and Kaluzna, M. 2010. Bacterial cankers caused by *Pseudomonas syringae* on stone fruit species with special emphasis on the pathovars *syringae* and *morsprunorum* race 1 and 2. Journal of Plant Pathology, 92 (1): 21–33.

Butler, M.I., Stockwell, P.A., Black, M.A., Day, R.C., Lamont, I.L. and Poulter, R.T.M. 2013. *Pseudomonas syringae* pv. *actinidiae* from recent outbreaks of kiwifruit bacterial canker belong to different clones that originated in China. PLoS ONE 8, e57464.

Carolis, E.D., Posteraro, B., Lass-Flo, C., Vella, A., Florio, A. R., Torelli, R., Girmenia, C., Colozza, C., Tortorano, A.M., Sanguinetti M. and Fadda, G. 2012. Species identification of Aspergillus, Fusariumand Mucoraleswith direct surface analysis by

matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clinic Microbiology Infect. 18:475-484.

Cetinkaya-Yildiz, R., Horuz, S., Karatas, A. and Aysan, Y. 2016. Identification and disease incidence of bacterial canker on stone fruits in the Eastern Mediterranean Region of Turkey. Acta Horticulturae, 1149:21-24.

Chandler, W.A. and Daniell, J.W. 1976. Relation of pruning time and inoculation with *Pseudomonas syringae* van Hall to short life of peach trees growing on old peach land. HortScience, 11:103-104.

Cirvilleri, G., Scuderi, G., Bonaccorsi, A. and Scortichini, M. 2006. Molecular characterization of *Pseudomonas syringae* pv. *syringae* strains from different host plants using fluorescent amplified fragment lenght polymorphism. Journal of Plant Pathology, 88(3): 327-331.

Crosse, J.E. 1966. Epidemiological relations of the pseudomonad pathogens of decidous fruit trees. Annu. Rev. Phytopathol., 14: 291-310.

Denny, T. P., Gilmour, M.N. and Selander, R.K. 1988. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. J. Gen. Microbiol., 134:1949–1960.

Dillon, M.M, Ruiz-Bedoya, T., Bundalovic-Torma, C., Guttman, K.M., Kwak, H., Middleton, M.A., Wang, P.W., Horuz, S., Aysan, Y. and Guttman, S. 2021. Comparative genomic insights into the epidemiology and virulence of plant pathogenic Pseudomonads from Turkey. Microbial Genomics, 7(7): 000585. doi: 10.1099/mgen.0.000585.

Excoffier, L. and Lischer, H.E.L. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources., 10: 564-567.

FAO, 2019. www.faostat.fao.org. 29\02\2021.

1 Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F. and Grimont, P.A.D. 1999. DNA
2 relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas*
3 *cannabina* sp. nov. (ex Sutic and Dowson 1959). International Journal of Systematic
4 Bacteriology, 49: 469-478.
5 Gašić, K., Prokić, A., Ivanović, M., Kuzmanović, N. and Obradović, A. 2012. Differentiation of
6 *Pseudomonas syringae* pathovars originating from stone fruits. Pesticides and Phytomedicine,
7 27 (3): 219–229
8 Gavrilovic, V., Zivkovic, S., Dolovac, N., Trkulja, N., Dolovac, E. P., Popovic, T. and Ivanovic,
9 D. 2013. *Pseudomonas syringae*-pathogen of sweet cherry in Serbia. Pestic. Phytomed, 27(2):
10 141-149.
11 Gilbert, V., Legros, F., Maraite, H. and Bultreys, A. 2009. Genetic analysis *of Pseudomonas*
12 *syringae* from Belgian fruit orchards reveal genetic variability and isolate-host relationships
13 within the pathovar *syringae*, and help idetify both races of the pathovar *morsprunorum*. Eur J
14 Plant Pathol., 124: 199-218.
15 Hulin, M.T., Mansfield, J.W., Brain, P., Xu, X., Jackson, R.W. and Harrison, R. J. 2018.
16 Characterization of the pathogenicity of strains of *Pseudomonas syringae* towards cherry and
17 plum. Plant Pathology, 67: 1177-1193.
18 Hwang, M.S.H., Morgan, R.L., Sarkar, S.F., Wang, P.W. and Guttman, D.S. 2005. Phylogenetic
19 characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. Appl.
20 Environ. Microbiol, 71:5182-5191.
21 Ilıcıc, R., Balaz, J., Stojsın, V. and Josic, D. 2016. Characterization of *Pseudomonas syringae*
22 pathovars from different sweet cherry cultivars by RAPD analysis. Genetika, 48 (1):285-295.

Ivanovic, Z., Stankovic, S., Zivkovic, S., Gavrilovic, V., Kojic, M. and Fira, D.2012. Molecular characterization of *Pseudomonas syringae* isolates from fruit trees and raspberry in Serbia. Eur J Plant Pathol, 134: 191-203.

Jones, A. 1971. Bacterial canker of sweet cherry in Michigan. Plant Dis. Rep., 55:961-965.

Kaluzna, M., Ferrante, P., Sobiczewski, P. and Scortichini, M. 2010. Characterization and genetic diversity of *Pseudomonas syringae* from stone fruits and hazelnut using repetiyive-PCR and MLST. Journal of Plant Pathology, 92(3): 781-787.

Kaluzna, M., Janse, J.D. and Young, J.M. 2012. Detection and identification methods and new tests as used and developed in the framework of COST 873 for bacteria pathogenic to stone fruits and nuts. Journal of Plant Pathology, 94(1):117-126.

Kara, M., Soylu, S., Kurt, Ş., Soylu, E.M. and Uysal, A. 2020. Determination of antagonistic traits of bacterial isolates obtained from apricot against green fruit rot disease agent *Sclerotinia sclerotiorum*. Acta Hortic., 1290: 135-142

Kennelly, M.M., Cazorla, F.M., Vicente, A., Ramos, C. and Sundin, G.W. 2007. *Pseudomonas syringae* diseases of fruit trees progress toward understanding and control. Plant Disease, 91(1):4-17.

King, E.D., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demeonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine, 44:301-7.

Konstantinos, T. and Tiedje, J.M. 2005. Towards a genome-based taxonomy for prokaryotes. Journal of Bacteriology, 185(18): 6258-6264.

Lattore, B.A. and Jones, A.L. 1979. *Pseudomonas morsprunorum*, the cause of bacterial canker sour cherry in Michigan, and its epiphytic association with *P. syringae*. Phytopathology, 69: 335-339.

1 Lelliott, R.A., Billing, E. and Hayward, A.C. 1966. A determinative cheme for the fluorescent
2 plant pathogenic pseudomonads. Journal of Applied Bacteriology, 29: 470–489.
3 Lelliott, R.A. and Stead, D.E. 1987. Methods for Diagnosis of Bacterial Diseases of Plants.
4 Blackwell Scientific Publications, Oxford, UK.
5 Librado, P. and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA
6 polymorphism data. Bioinformatic, 25(11):1451-1452.
7 Little, E.L., Bostock, R.M. and Kırkpatrick, B.C. 1998. Genetic characterizaiton of *Pseudomonas*
8 *syringae* pv. *syringae* strains from stone fruits in California. Applied and Environmental
9 Microbiology, 64(10): 3818-3823.
10 Lo, T., Koulena, N., Seto, D., Guttman, D. S. and Desveaux, D. 2017. The HopF family
11 of *Pseudomonas syringae* type III secreted effectors. Mol. Plant Pathol., 18:457–468.
12 Louws, F.J., Fulbright, D.W., Stephens. C.T. and DeBruijn, F.J. 1994. Specific genomic
13 fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains
14 generated with repetitive sequences and PCR. Appl Environ Microbiol, 60:2286-2295.
15 Martin-Sanz, A., de la Vega, M.P., Murillo, J. and Caminero, C. 2013. Strains of *Pseudomonas*
16 *syringae* pv. *syringae* from pea are phylogenetically and pathogenically diverse.
17 Phytopathology, 103(7): 673-681.
18 Mitchell, R.E. 1991. Implications of toxins in the ecology and evolution of plant pathogenic
19 microorganisms: bacteria. Experientia 47:791–803.
20 Mohammadi, M., Ghasemi, A. and Rahimian, H. 2001. Phenotypic characterization of Iranian
21 strains of *Pseudomonas syringae* pv. *syringae* van Hall, the causal agent of bacterial canker
22 disease of stone fruits trees. Journal of Agricultural Science and Technology, 3: 51−65.

Moragrega, C., Llorente, I., Manceau, C. and Montesinos, E. 2003. Susceptibility of European pear cultivars to *Pseudomonas syringae* pv. *syringae* using immature fruit and detached leaf. European Journal of Plant Pathology, 109:319-326.

Natalini, E., Rossi, M., Barionovi, D. and Scortichini, M. 2006. Genetic and pathogenic diversity of *Pseudomonas syringae* pv. *syringae* isolates associated with bud necrosis and leaf spot of pear in a single orchard. Journal of Plant Pathology, 88(2):219-223.

Oksel, C. and Mirik, M. 2021. Identification of causal disease agent(s) of cherry bacterial canker in Marmara Region of Turkey. Currrent Trends in Natural Sciences, 10(19): 368-374.

Özçağıran, R., Ünal, A., Özeker, E. and İsfendiyaroğlu, M. 2005. Ilıman iklim meyve türleri. İçinde: Sert Çekirdekli Meyveler Cilt-I, 553. edn. Ege Üniversitesi Ziraat Fakültesi Yayınları, İzmir, s 229.

Popovic, T., Menkovic, J., Prokic, A., Zlatkovic, N. and Obradovic, A. 2021. Isolation and characterization of *Pseudomonas syringae* isolates afecting stone fruits and almond in Montenegro. Journal of Plant Diseases and Protection, 128:391-405.

Rademaker, J.L.W. and De Bruijn, F.J. 1997. Characterization and Classification of Microbes by Rep-PCR Genomic Fingerprinting and Computer Assisted Pattern Analysis. In: Caetano-Anollés, G. and Gresshoff, P.M., Eds., DNA Markers: Protocols, Applications and Overviews, John Wiley & Sons, Hoboken, 151-171.

Ruinelli, M., Blom, J., Smits, T.H.M. and Pothier, J. 2019. Comparative Genomics and pathogenicity potential of Members of *Pseudomonas syringae* species complex on Prunus spp. BMC Genomics, 20:172.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. A laboratory manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory Press,1659 p.

Sarkar, S.F. and Guttman, D.S. 2004. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. Applied and Environmental Microbiology, 1999-2012.

Sawada, H. and Fujikawa, T. 2019. Genetic diversity of *Pseudomonas syringae* pv. *actinidiae*, pathogens of kiwifruit bacterial canker. Plant Pathology, 68(7): 1235-1248.

Schaad, N.W., Jones, J.B. and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. The American Phytopathological Society, St. Paul, MN., USA.

Scholz-Schroeder, B.K., Hutchison, M.L., Grgurina, I. and Gross, D.C. 2001. The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. syringae strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis. Mol. Plant Microbe Interact*.,* 14:336–348.

Scortichini, M., Marchesi, U., Dettori, M.T. and Rossi, M.P. 2003. Genetic diversity, presence of the syrB gene, host preference and virulence of *Pseudomonas syringae* pv. *syringae* strains from woody and herbaceous host plants. Plant Pathol., 52:277-286.

Sisto, A., Cipriani, M.G., Tegli, A., Cerboneschi, A.M., Stea, G. and Santili, E. 2007. Genetic characterizaiton by fluorescent AFLP of *Pseudomonas savastanoi* pv. *savastanoi* strains isolated from different host species. Plant Pathology, 56: 366-372.

Sorensen, K.N., Kim, K.H. and Takemoto, J.Y. 1998. PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. Applied and Environmental Microbiology, 64 (1): 226−230.

Spotts, R. A., Wallis, K. M., Serdani, M. and Azarenko, A.N. 2010. Bacterial canker of sweet cherry in Oregon-infection of horticultural and natural wounds and resistance of cultivar and rootstock combination. Plant Disease, 93: 345-350.

Stead, D.E., Simkins, S.A., Weller, S.A., Hennessy, J., Aspin, A., Stanford, H., Smith, N.C. and Elphinstone, J.G. 2003. Classification and identification of planth pathogenic Pseudomonas species by REP-PCR derived genetic fingerprints. *Pseudomonas syringae* and related pathogens. Kluwer Academic Publisher.

Sulikowska, M. and Sobiczewski, P. 2008. Pseudomonas spp. isolated from stone fruits trees in Poland. Zemdirbyste-Agriculture, 95(3):166-170.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30:27, 252-729.

Uysal, A., Kurt, Ş., Soylu, S., Soylu, E.M. and Kara, M. 2019. Identification of microorganism species in leafy vegatables using MALDI-TOF MS tecnique. YYU Journal of Agricultural Science, 29(4):595-602.

Versalovic, J., Schneider, M., de Bruijn, F.J. and Lupski, J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based PCR (rep-PCR). Methods Mol Cell Biol, 5:25-40.

Vicente, J.G., Alves, J.P., Russell, K. and Roberts, S.J. 2004. Identification and discrimination of *Pseudomonas syringae* isolates from wild cherry in England. European Journal of Plant Pathology, 110(4): 337-351.

Vicente, J. and Roberts, S. 2007. Discrimination of *Pseudomonas syringae* isolates from sweet and wild cherry using rep-PCR. European Journal of Plant Pathology, 117:383-392.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A. and Harayama, S. 2000. Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology, 146:2385–2394.

Young, J.M. 2010. Taxonomy of *Pseudomonas syringae*. Journal of Plant Pathology, 92(1):5-14.

1 Ziegler, D., Mariotti, A., Pflüger, V., Saad, M., Vogel, G., Tonolla, M. and Parret, X. 2012. In
2 Situ identification of plant-invasive bacteria with MALDI-TOF Mass Spectrometry. PloS
3 ONE, 7:5

Tables

Table 1. *Pseudomonas syringae* pv. *syringae* isolates used in the present study.

Isolate	**Host**	**Year**	**Location**	**Gram staining**	**Fluorescent pigment on PSF**	**Pathogenicity tests**	**LOPAT**	**GATTa**	***syrB***	***cfl***	**Score value of MALDI-TOF MS**	**Result**
Reference isolate (*Pss* Naip 1)	Sweet cherry (*P. avium* cv. 0900 Ziraat)	**2013**	**Turkey (Tekirdag/ Naip)**	**-**	+	+	+---+	++--	+	**-**	2.631	***Pss***
C101	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2017	Turkey (Tekirdag/ Naip)	-	+	+	+---+	++--	+	-	2.289	***Pss***
C102	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2017	Turkey (Balikesir)	-	+	+	+---+	++--	+	-	2.610	***Pss***
C103	Sweet cherry (*P. avium* cv.	2018	Turkey (Canakkale/	-	+	+	+---+	++--	+	-	2.240	***Pss***

	0900 Ziraat)		Lapseki)									
C104	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2017	Turkey (Canakkale/ Lapseki)	-	+	+	+---+	++--	+	-	2.312	***Pss***
C105	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Canakkale/ Bayramic)	-	+	+	+---+	++--	+	-	2.282	***Pss***
C106	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Canakkale/ Lapseki)	-	+	+	+---+	++--	+	-	2.374	***Pss***
C107	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Tekirdag/ Marmara ereglisi)	-	+	+	+---+	++--	+	-	2.323	***Pss***
C108	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Istanbul/ Selimpaşa)	-	+	+	+---+	++--	+	-	2.241	***Pss***
C109	Sweet cherry (*P. avium* cv. 0900 ziraat)	2018	Turkey (Kırklareli)	-	+	+	+---+	++--	+	-	2.193	***Pss***

C110	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Tekirdag/ Yeniciftlik)	-	+	+	+---+	++--	+	-	2.031	***Pss***
C112	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Bursa/ Osmangazi)	-	+	+	+---+	++--	+	-	2.123	***Pss***
C113	Sweet cherry (*P. avium* cv. 0900 Ziraat)	2018	Turkey (Tekirdag/ Kirazli)	-	+	+	+---+	++--	+	-	2.106	***Pss***

+: positive reaction, -:negative reaction-

14 Table 2. List of primers used in this study.

Method	Region/ Gene/ Primer ID	Direction	Primer (5′-3′)	Tm (°C)
rep-PCR	ERIC	F	ATGTAAGCTCCTGGGGATTCAAC	52
		R	AAGTAAGTGACTGGGGTGAGCG	
	REP	F	IIIICGICGICATCIGGC	44
		R	ICGICTTATCIGGCCTAC	
	BOX	NA	CTACGGCAAGGCGACGCTGACG	53
MLST	DNA gyranase (gyrB)	F	MGGCGGYAAGTTCGATGACCAYTC	63
		R	TRAKTBKCAGTCARACCTTCRCGSGC	
	Sigma factor 70 (rpoDp)	F	AAGGCGARATCGAAATCGCCAAGCG	63
		R	GGAACWKGCGCAGGAAGTCGGCACG	
	Sigma factor 70 (rpoDs)	F	AAGCGAATCGAAGAAGGCATYCGTG	63
		R	GGAACWKGCGCAGGAAGTCGGCACG	
	Citrate synthese (gltA)	F	GCCTCBTGCGAGTCGAAGATCACC	64
		R	CTTGTAVGGRCYGGAGAGCATTTC	

15 Table 3. The NCBI GenBank accession numbers of *P. s.* pv. *syringae* isolates used in this study.

Isolate	**GenBank Accession Number**			
	gyrB	***rpoDs***	***rpoDp***	***GltA***
C101	MT508572	MT521868	MT508584	MT508560
C102	MT508573	MT521878	MT508585	MT508561
C103	MT508574	MT521869	MT508586	MT508562
C104	MT508575	NA*	NA*	MT508563
C105	MT508576	MT521870	MT508587	MT508564
C106	MT508577	MT521871	NA*	MT508565
C107	MT508578	MT521872	MT508588	MT508566
C108	MT508579	MT521873	MT508589	MT508567
C109	MT508580	MT521874	NA*	MT508568
C110	MT508581	MT521875	MT508590	MT508569
C112	MT508582	MT521876	MT508591	MT508570
C113	MT508583	MT521877	MT508592	MT508571

16 * Sequencing was not successful

Table 4. Nucleotide data exploration of *gyrB*, *rpoDs*, *rpoDp*, and *gltA* genes as well as the concatenated sequences of those.

Gene	**Number of sequences considered**	**Conserved sites**	**Variable sites**	**Parsimony informative sites**	**Singleton sites**	**Overall mean distance (diversity)**
gyrB	12	627/640 95.24%	13/640 2.03%	11/640 1.71%	2/640 0.31%	0.007
rpoDs	11	530/540 98.14%	10/540 1.85%	9/540 1.66%	1/540 0.18%	0.05
rpoDp	9	556/591 94.07%	23/591 3.81%	6/591 1.01%	12/591 2.03%	0.011
gltA	12	964/998 96.60%	24/988 2.42%	24/988 2.42%	0	0.012
gyrB+rpoDs+ rpoDp+gltA	44	2677/2765 96.81%	70/2765 2.53%	50/2765 1.80%	20/2765 0.72%	0.010

Figures

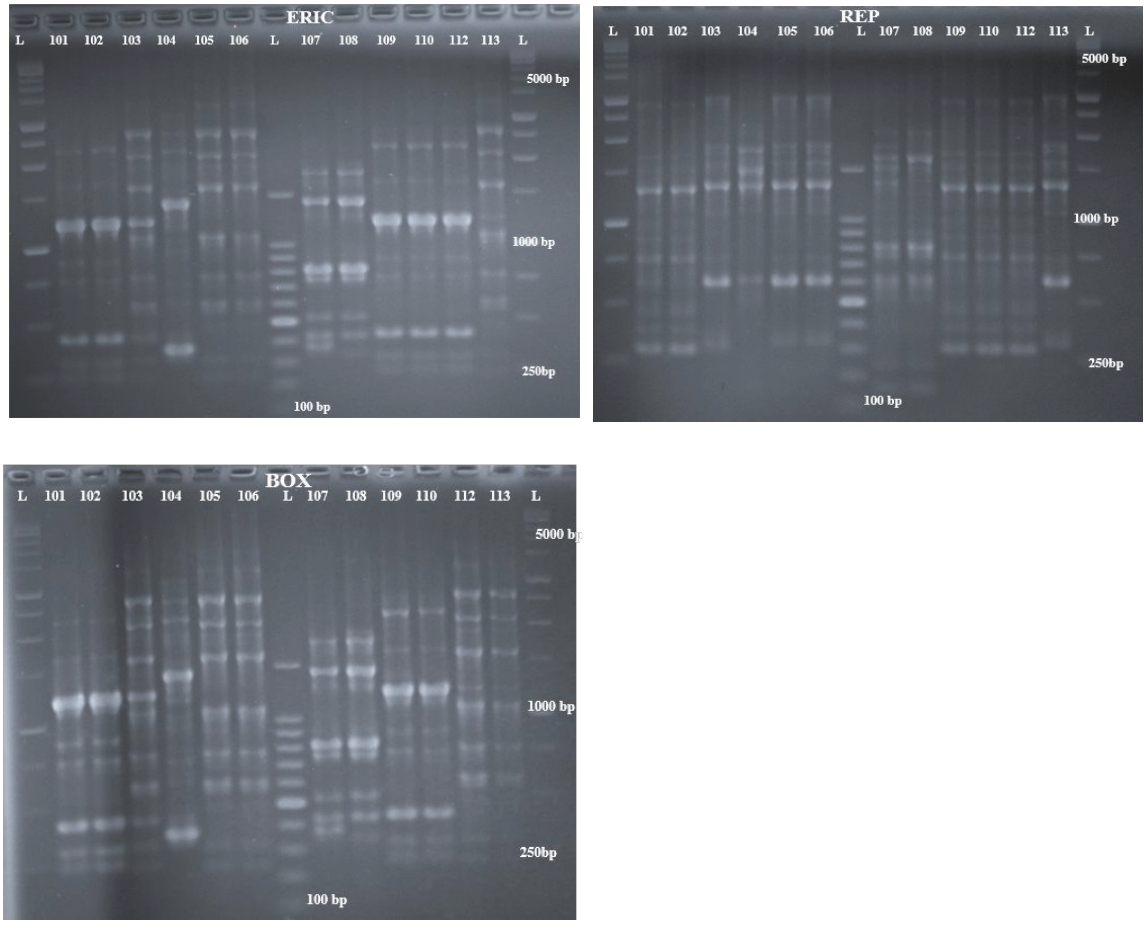


Fig. 1. PCR fingerprinting patterns of rep-PCR using ERIC, REP, and BOX-PCR of *Pseudomonas syringae* pv. *syringae* isolates obtained from sweet cherry. The codes above the lanes refer to sample ID (Table 1).

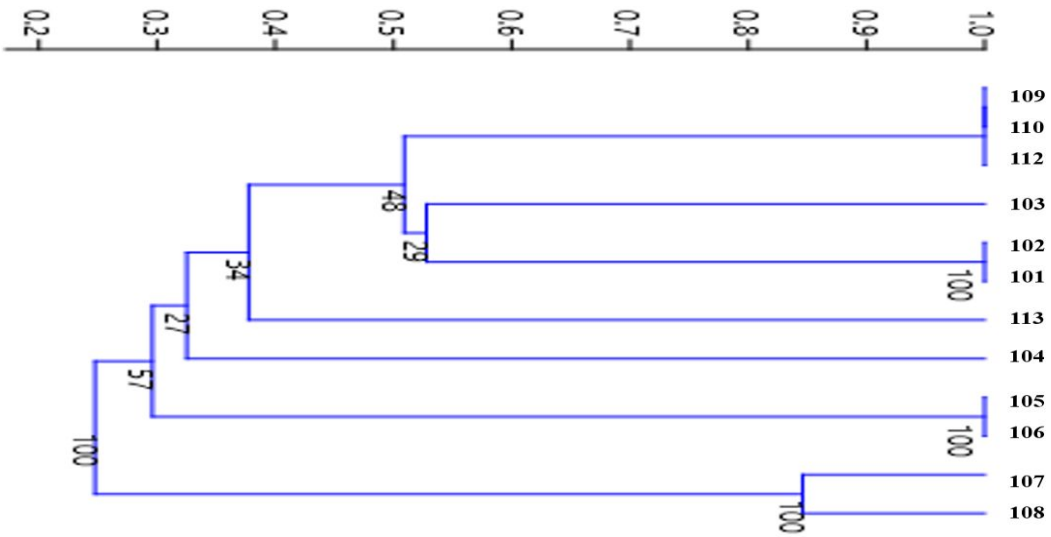
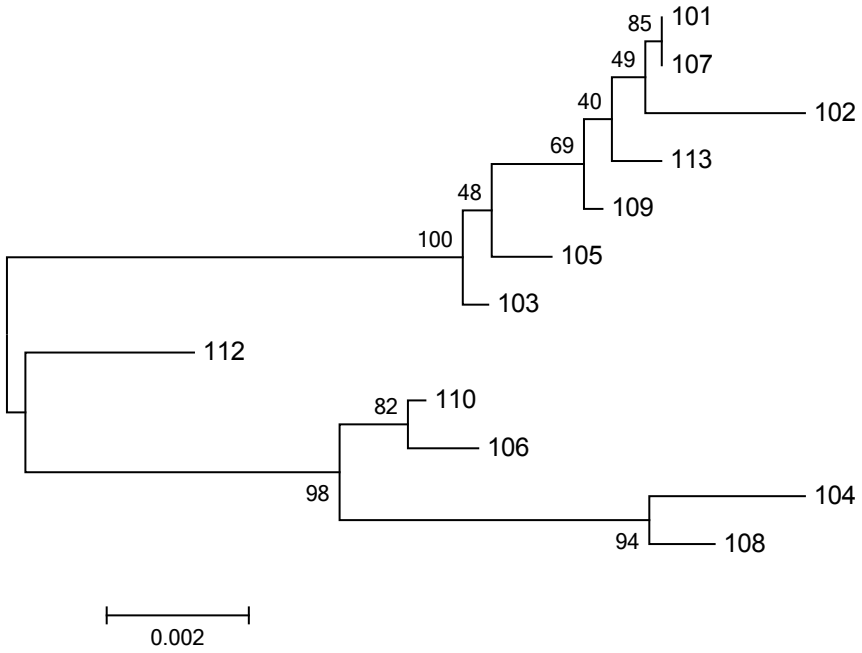


Fig. 2. Dendrogram of genetic similarity of 12 *Pseudomonas syringae* pv. *syringae* isolates obtained from the sweet cherry in different regions of Turkey. The combined data set from REP-, ERIC-, and BOX-PCR was used using the UPGMA method based on Jaccard's similarity index. Numbers close to branches indicate 1,000 replications of bootstrap test and the codes refer to sample ID (Table 3).



45

46 **Fig. 3.** Neighbor-joining phylogenetic tree based on the concatenated sequences of *gyrB*, *rpoDs*,

47 *rpoDp*, and *gltA* from *Pseudomonas syringae* pv. *syringae* isolates. Numbers close to branches

48 indicate 1000 replications of bootstrap test and the codes refer to sample ID (Table 3).

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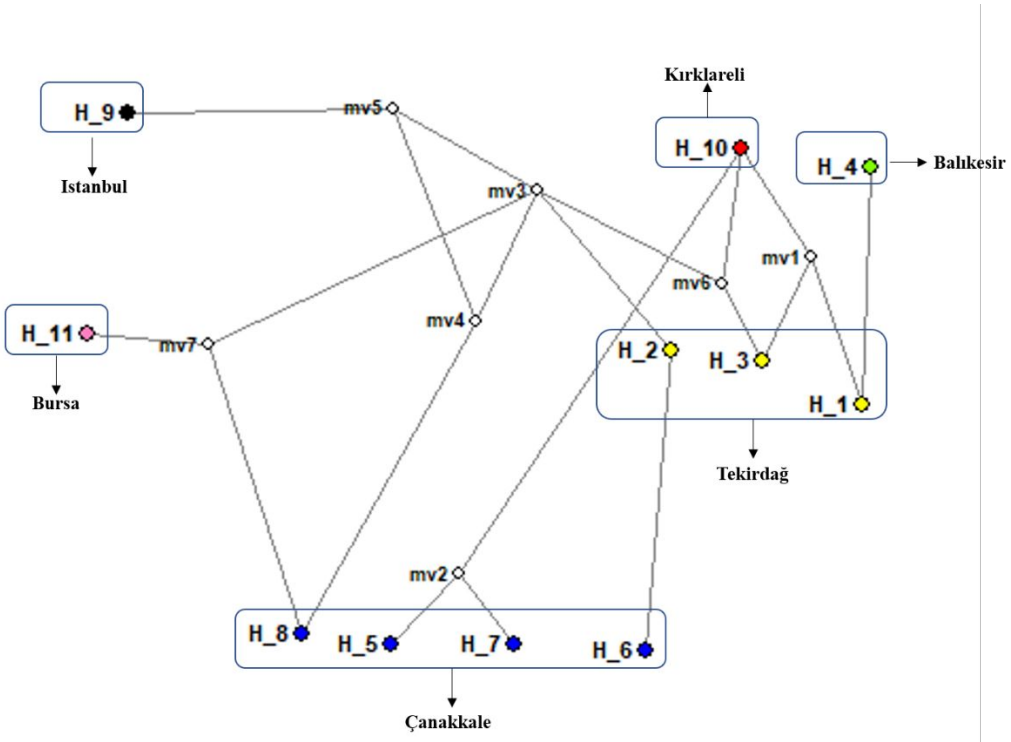

52

53 **Fig. 4.** The median-joining haplotype network using concatenated sequences of *gyrB, rpoDp,*

54 *rpoDs*, and *gltA* genes.

55

56 Supplementary Table 1. Survey details.

Number	**Location**	**Symptoms**	**Host**	**Cultivar**	**Tree age**	**Year of isolation**
1	Tekirdağ/Naip	Branch necrosis	*Prunus avium*	0900 Ziraat	8-10	2016
2	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2016
3	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2016
4	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2016
5	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2016
6	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2016
7	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8	2017
8	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
9	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
10	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2017
11	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
12	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
13	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
14	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
15	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017

16	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
17	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
18	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
19	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2107
20	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
21	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2107
22	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
23	Tekirdağ/Kirazlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2107
24	Tekirdağ/Çınarlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
25	Tekirdağ/Çınarlı köyü	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2107
26	Tekirdağ/Çınarlı köyü	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2017
27	Tekirdağ/Çınarlı köyü	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2107
28	Tekirdağ/Çınarlı köyü	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2017
29	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
30	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2107
31	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2017
32	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2017
33	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
34	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017

35	Tekirdağ/Kumbağ	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
36	Tekirdağ/Kumbağ	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2017
37	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8	2017
38	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8	2017
39	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8	2017
40	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8	2017
41	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8	2017
42	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8	2017
43	Balıkesir/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2017
44	Balıkesir/Merkez	Blossom blast	*P. avium*	0900 Ziraat /Lambert	8-12	2017
45	Balıkesir/Merkez	Blossom blast	*P. avium*	0900 Ziraat /Lambert	8-12	2017
46	Balıkesir/Merkez	Blossom blast	*P. avium*	0900 Ziraat /Lambert	8-12	2017
47	Balıkesir/Merkez	Bud necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2017
48	Bursa/Gürsu	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2017
49	Bursa/Gürsu	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2017
50	Bursa/Gürsu	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2017
51	Bursa/Gürsu	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2017
52	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2017
53	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2017

54	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2017
55	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2017
56	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2017
57	Çanakkale/Lapseki	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2017
58	Kırklareli/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-7	2017
59	Kırklareli/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-7	2017
60	Kırklareli/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-7	2017
61	Kırklareli/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-7	2017
62	Kırklareli/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-7	2017
63	İstanbul/Selimpaşa	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-6	2017
64	İstanbul/Selimpaşa	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-6	2017
65	İstanbul/Selimpaşa	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-6	2017
66	İstanbul/Selimpaşa	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-6	2017
67	İstanbul/Selimpaşa	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	5-6	2017
68	Edirne/Merkez	Branch necrosis	*P. avium*	0900 Ziraat	6-8	2017
69	Edirne/Merkez	Branch necrosis	*P. avium*	0900 Ziraat	6-8	2017
70	Edirne/Merkez	Branch necrosis	*P. avium*	0900 Ziraat	6-8	2017
71	Kocaeli/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	7-8	2017
72	Kocaeli/Merkez	Leaf spot	*P. avium*	0900 Ziraat /Lambert	7-8	2017

73	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
74	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
75	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
76	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
77	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
78	Tekirdağ/Yeniçiftlik	Branch necrosis	*P. avium*	0900 Ziraat	8	2018
79	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
80	Tekirdağ/Marmaraereğlisi	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
81	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
82	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
83	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
84	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
85	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
86	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
87	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
88	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
89	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
90	Tekirdağ/Naip	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
91	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018

92	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
93	Tekirdağ/Barbaros	Branch necrosis	*P. avium*	0900 Ziraat	8-10	2018
94	Bursa/Yenişehir	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
95	Bursa/Yenişehir	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
96	Bursa/Yenişehir	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
97	Bursa/Yenişehir	Leaf spot	*P. avium*	0900 Ziraat /Lambert	8-12	2018
98	Bursa/Yenişehir	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
99	Bursa/Yenişehir	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
100	Bursa/Osmangazi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
101	Bursa/Osmangazi	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-12	2018
102	Çanakkale/Bayramiç	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2018
103	Çanakkale/Bayramiç	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2018
104	Çanakkale/Bayramiç	Branch necrosis	*P. avium*	0900 Ziraat /Lambert/Klasik	10-15	2018
105	Yalova/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
106	Yalova/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018
107	Yalova/Merkez	Branch necrosis	*P. avium*	0900 Ziraat /Lambert	8-10	2018

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