# PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *PSEUDOMONAS SAVASTANOI* PV. *SAVASTANOI* CAUSING OLIVE KNOT DISEASE IN TURKEY

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**Abstract.** Olive knot disease caused by *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) is one of the major diseases influencing olive (*Oleae europaea* L.) production in Turkey. The disease incidence rate was found to range between 4 and 80% according to the 2015 and 2018 surveys. A total of 67 isolates were recovered from 7 symptomatic Turkish olive cultivars on a semi-selective medium, PVF-1, and then identified as *Psv* by biochemical and molecular tests. The isolates produced characteristic gall symptoms on olive plants and were consistently re-isolated. Fatty acid methyl ester (FAME) analysis indicated that the major fatty acid components were oleic acid (18:1), palmitoleic acid (16:1), and palmitic acid (16:0), and also clustered the olive strains into 2 groups. The repetitive element palindromic PCR (rep-PCR) primer, the BOXA1R primer, produced the discriminatory profile, with amplicon sizes ranging from ~ 200 bp to 2 kb, and categorized the olive strains into 2 separate groups. Pulsed-Field Gel Electrophoresis (PFGE) differentiated the olive *Psv* isolates into 3 discrete haplotype groups after the genomic DNA was digested with *Spe*I. This is the first study using PFGE to determine the genetic diversity of the *Psv* olive population.
**Keywords:** *FAME, Rep-PCR, olive knot, PFGE, Psv*

## Introduction

The olive tree (*Olea europaea* L.) is a standout amongst the most significant and transcendent organic product trees found in western and focal Italy and Spain, southern Morocco and Tunisia, and western Turkey, and Greece (Loumou and Giourga, 2003). It serves as a source of edible fruit and oil for millions of people in various parts of Turkey. At present, global olive oil production exceeds 2,500,000 tons. In 2015, the aggregate production by the member states of the International Olive Oil Council (IOC) was 2,964,500 tons, 94% of the global production. EU production was 2,322,000 tons, whereas the individual IOC member state production showed Spain to be leading with 1,401,600 tons, followed by Italy with 474,600 tons, and then by Greece with 320,000 tons. Turkey produced 143,000 tons, Tunisia 140,000 tons, Morocco 130,000 tons, Algeria 83,500 tons, and Iran 5,000 tons (International Olive Oil Council, 2015). Olive knot disease is perhaps the earliest plant disorder to be specifically reported in ancient times, and is regarded as one of the most critical diseases that affects olive trees.

Olive knot disease is characterised by the production of hyperplastic galls on several plant parts (Nester and Kosuge, 1981). The disease is a serious threat to olive production in the Mediterranean basin, including Turkey, where several climatic conditions including rain, wind, humidity, and temperature favour pathogen


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dissemination, growth, development, and infection processes. In Turkey, the disease is known to be infect olive (*Olea* sp.), oleander (*Nerium oleander*), fontanesia (*Fontanesia phillyreoides*), myrtle (*Myrtus communis*), and jasmine (*Jasminium officinale*) in Çanakkale, Muğla, Antalya, Samsun, Şanlıurfa, Bursa, Izmir, Tekirdağ, Adana, Mersin, and Hatay provinces (Basım and Ersoy, 2000; Tatlı and Benlioğlu 2004; Kavak and Üstün 2009; Mirik et al., 2011).

The development of olive knots relies on the ability of *Psv* to synthesise and secrete cytokinins and indolacetic acid (IAA) (Surico et al., 1985; Perez-Martínez et al., 2007; Quesada et al., 2010) as well as the functionality of its Type Three Secretion System (Sisto et al., 2004; Perez-Martinez et al., 2010; Tegli et al., 2011). Moreover, the capacity of *Psv* to actuate olive knot arrangement depends on the quorum sensing intercellular communication system moderated by N-acyl homoserine lactone (Hosni et al., 2011). The disease affects the yield and vegetative parts of the olive plants, and quality of olive oil (Schroth et al., 1973).

Members of *P. syringae*, including *Psv*, have been previously identified based on plasmid fingerprints (King, 1989), fatty acid fingerprinting (Stead, 1992), and analysis of protein (Van Zyl and Steyn, 1990). Recently, DNA profiling techniques such as Restriction Fragment Length Polymorphism (RFLP) (Manceau and Horvais, 1997), Amplified Fragment Length Polymorphism (AFLP), (REP)-REP1-1/REP2-1 (Sharples and Lioyd, 1990), Enterobacterial Repetitive Intergenic Consensus (ERIC)-ERIC1R/ERIC2 (Hulton et al., 1991), and BOXA1R (Louws et al., 1994), Randomly Amplified Polymorphic DNA (Krid et al., 2009), Pulsed-field gel electrophoresis (PFGE) (Chu et al., 1986), Multi Locus Sequence Typing (MLST) analysis of housekeeping genes (Berge et al., 2014), and a set of primers (Guilbaud et al., 2016) have been applied in genotyping and investigating strains and pathovars of *P. syringae*. The whole genome of *Psv* has also been sequenced and annotated (Rodriguez-Palenzuela et al., 2010). Although there are reports of the presence and identification of *Psv* in west Mediterranean region of Turkey (Basım and Ersoy 2000, 2001), few studies have been conducted to characterize this pathogen in detail.

The objective of this study was to evaluate the phenotypic and genotypic features of the *Psv* olive population in west Mediterranean region of Turkey by gas chromatographic analysis of fatty acid methyl esters (GC-FAME), repetitive element palindromic PCR (rep-PCR), and PFGE. To our knowledge, this is the first study of using PFGE to determine the genetic diversity of the *Psv* olive population.

## Materials and methods

### *Sample collection*

Samples were collected from olive trees showing symptoms of suspected knot disease by random sampling of different olive orchards in the districts of Antalya Center (7 orchards; 82 olive trees), Serik (3 orchards; 49 olive trees), Aksu (4 orchards; 35 olive trees), Kaş (6 orchards; 96 olive trees) and Döşemealtı (3 orchards; 58 olive trees) in the Western Mediterranean district of Turkey during May–August of 2015 and May–August of 2018. Samples from different local cultivars of olive trees such as Gemlik, Memeli, Ayvalık, Edinciksu, Edremit, Kan, and Memecik were collected for bacterial isolation. Approximately 320 olive trees in 23 orchards in total were surveyed. The samples were packaged and transported to the Department of Plant Protection of Akdeniz University, where the putative pathogen was isolated. *Psv* isolates (*Pss*I2,


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*Pss*I7, *Pss*I21, *Pss*I24, *Psn*9) obtained from surveys in Antalya province of Turkey in 2000 (Basım and Ersoy, 2000) were also included in this study (*Table 2*). The Myrtus strain, *Pss*I24, and Nerium strain, *Psn*9, were used for clustering. The disease rates within the olive plantations from which the contaminated samples were collected were assessed utilizing the equation as defined by Bansal et al. (1994):

$$\text{Disease incidence } (DI)\ (\%) = (\text{Number of diseased plants} / \text{Total number of plants}) \times 100$$

### *Isolation and identification of bacteria*

Bacterial isolation was carried out on olive knots present on two-year-old twigs. Plant surfaces were disinfected using paper towel saturated with 96% ethanol; the decayed sections within the hyperplastic knot tissues were then cut off and grounded in sterile phosphate buffer (0.05 M $Na_2HPO_4$, 4.26 g; $K_2HPO_4$, 2.72 g; 1000 ml sterile $dH_2O$). A loop of bacterial suspension was streaked onto semi-selective PVF-1 medium (glycerol, 10 ml; sucrose, 30 g; casamino acid, 2.5 g; $K_2HPO_4.3H_2O$, 1.96 g; $MgSO_4.7H_2O$, 0.4 g; SDS, 0.4 g; agar, 16 g; 1000 ml $dH_2O$; pH 7.1) (Surico and Lavermicocca, 1989), King's B medium (KB; protease peptone, 20 g; Bacto agar, 20 g; glycerol, 10 ml; $K_2HPO_4$, 1.5 g; $MgSO_4$, 1.5 g; 1000 ml $dH_2O$, pH 7.2) (King et al., 1954) and Nutrient Sucrose Agar (NSA) (Nutrient Broth, 4.8 g; Bacto agar, 9 g; sucrose, 30 g; 600 ml $dH_2O$) (Surico and Lavermicocca, 1989). The *Psv* isolates utilized in this study are listed in *Table 2*. The bacteria were incubated at 28 °C on PVF-1, KB, and NSA media. Bacterial fluorescent pigment production was examined under UV light at 364 nm. In all, 67 isolates identified as *Psv* were recovered, then freezed in glycerol (30%) at -86 °C for long term storage and further analysis.

Putative *Psv* isolates were identified by several biochemical tests, including LOPAT, according to Schaad et al. (2001). The tests were carried out using flat, greyish-white, irregular margins or semi-translucent colonies grown on PVF-1 medium. The identity of the *Psv* isolates was confirmed by GC-FAME analysis (Stead, 1992) and PCR. The *Psv* isolates were identified directly from bacterial suspension as well as from purified genomic DNA by PCR utilizing primers, IAALN1/IAALN2 (Pelyaver et al., 2000) and PSS1/PSS2; PSS3/PSS4 (Basım and Ersoy, 2001). The polymerase chain reaction (PCR) conditions are given in *Table 1*.

### *Pathogenicity test*

For putative *Psv* isolates, pathogenicity tests were carried out by inoculating the stems of a one-year-old Gemlik olive cultivar. The bark of the stem was wounded with a sterilized needle dipped in bacterial suspension, which contained ~$10^8$ CFU/ml, and the wounds were then covered with Parafilm for 3 days. The inoculated olive plants were kept in a controlled-room at $25 \pm 2$ °C and 80-85% RH and monitored for symptom development according to Surico and Lavermicocca (1989). Olive plants similarly treated with reference strains or sterile $dH_2O$ were utilized as positive and negative controls, respectively.

### *GC-FAME analysis*

GC-FAME analyses were performed on each *Psv* isolate as stated in the manufacturer's specifications to determine the phenotypic characteristic of the isolates. The *Psv* isolates were growth on tryptic soy broth agar at 27 °C for 24 h. The each *Psv*


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isolate (a loopful) was blended within a glass tube containing 1.2 N NaOH in methanol: $H_2O$. The tubes were shortly vortexed, kept in a bubbling water bath for 5 min. The tubes firmly vortexed once more for 10 s and were exchanged to the bubbling water bath for 30-min warming to finish reaction. Two ml methylation arrangement (325 ml 6.0 N hydrochloric acid, 275 ml methyl solution) was included to the tubes after the tubes were cooled to room temperature. The samples were without further ado vortexed, warmed at 80 °C for 10 min. The samples were cooled quickly on ice, and afterward 1.25 ml the extraction arrangement (N-hexane/methyl tert-butyl ether (1:1; v/v) was included. The samples were tenderly blended utilizing a tube revolver for 10 min. The under aqueous phases of the samples were discarded by a micropipette. Three milliliters of the sample clean up solution (10.8 g NaOH dissolved in 900 ml $H_2O$) was included into the sample, and tenderly blended for 5 min. And after that ~ 2/3 of natural period of the examples was moved into a dull GC vial. These final extracts were analysed with the HP 6980 GC System (Agilent Technologies, CA, USA), and the MIDI system (Microbial ID Inc., USA) with a 25 m × 0.2 mm silica capillary column was performed. The 67 isolates of *Psv* were identified, and phenotypically characterized based on FAME composition by dendrogram analysis using the MIDI software version 6.0.

***Table 1.*** *PCR primers used in this study*

| Locus | Primers | Sequence (5'→3') | Size (bp) | References | Conventional PCR conditions |
|---|---|---|---|---|---|
| *Ptz* | PSS1 | TGGGGTGCTACTTGTACCGGA | 684 | Basım and Ersoy (2001) | 5 min at 95 °C, followed by 35 cycles, 30 s at 95 °C, 30 s at 62 °C, 45 s at 72 °C, 5 min at 72 °C |
| | PSS2 | CCGTGTACTACGTTCAGCGAG | | | |
| *iaa*L | PSS3 | CAGGACTTCAGAACCCACGT | 1064 | Basım and Ersoy (2001) | 5 min at 95 °C, followed by 35 cycles, 30 s at 95 °C, 30 s at 62 °C, 45 s at 72 °C, 5 min at 72 °C |
| | PSS4 | CGGTCGATGATGTAGAGCAT | | | |
| *iaa*L | IAALF | GGCACCAGCGGCAACATCAA | 454 | Pelyalver et al. (2000) | 5 min at 94 °C, followed by 35 cycles, 30 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C, 5 min at 72 °C m |
| | IAALR | CGCCCTCGGAACTGCCATAC | | | |
| BOX-PCR | BOXA1R | CTACGGCAAGGCGACGCTGACG | | Louws et al. (1994) | 7 min at 95 °C, followed by 30 cycles, 1 min at 94 °C, 1 min at 53 °C, 8 min at 65 °C, 15 min at 65 °C |

### *DNA extraction*

Genomic DNA from *Psv* isolates was extracted based on a modified CTAB method (Doyle and Doyle, 1990), and was dissolved in 50 µl TE buffer. The concentration of DNA was adjusted to 100 ng/µl with TE, using a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and after which the DNA solution was stored at 4 °C.

### *Rep-PCR*

Evaluation of genetic differences among *Psv* was performed by Rep-PCR using primer based on repetitive extragenic palindromic (REP) sequences, BOXA1R (Louws


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et al., 1994). The reaction mixture consisted of 4 µl DNA, 27.8 µl dd$H_2O$, 5 µl 10x *Taq* buffer, 3 µl $MgCl_2$, 8 µl 0.1 mM dNTPs, and 3.5 U *Taq* Pol. The BOX-PCR conditions are given in *Table 2*. PCR products (10 µl) were fractionated by gel electrophoresis (75 V, for 7 h) on 0.5% standard and 0.5% NuSieve agarose. A 1 kb DNA size marker (Fermantas, Vilnius, Lithuania) was used. The agarose gels were stained with ethidium bromide (0.5 µg/ml) for 20 min., and pictured using a imaging system (Vilber Lourmat transilluminator, France). The outcomes were analysed by Bio-gene gel analysing software (VilberLourma, France). UPGMA (Sneath and Sokal, 1973) and Dice coefficient (2a/(2a + b + c) (Dice, 1945) were utilized for the DNA fingerprint matching and dendrogram analyses.

***Table 2.*** *Bacterial isolates used for the study*

| Haplotype groups | Strain designation | Host plant of isolation/cultivar | Geographical origin | Isolation date | Haplotype groups | Strain designation | Host plant of isolation/cultivar | Geographical origin | Isolation date |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Pss* 1 | Olive/Gemlik | Aksu | 2015 | 2 | *Pss* 13 | Olive/Gemlik | Antalya | 2015 |
| 1 | *Pss* 2 | Olive/Gemlik | Aksu | 2015 | 2 | *Pss* 16 | Olive/Kan | Antalya | 2015 |
| 1 | *Pss* 3 | Olive/Kan | Aksu | 2015 | 2 | *Pss* 20 | Olive/Edinciksu | Kaş | 2015 |
| 1 | *Pss* 4 | Olive/Gemlik | Serik | 2015 | 2 | *Pss* 22 | Olive/Edremit | Kaş | 2015 |
| 1 | *Pss* 6 | Olive/Gemlik | Dösemaltı | 2015 | 2 | *Pss* 24 | Olive/Memecik | Kaş | 2015 |
| 1 | *Pss* 7 | Olive/Kan | Dösemaltı | 2015 | 2 | *Pss* 25 | Olive/Kan | Antalya | 2015 |
| 1 | *Pss* 9 | Olive/Kan | Dösemaltı | 2015 | 2 | *Pss* 26 | Olive/Gemlik | Antalya | 2015 |
| 1 | *Pss* 10 | Olive/Edinciksu | Kaş | 2015 | 2 | *Pss* 29 | Olive/Ayvalık | Antalya | 2015 |
| 1 | *Pss* 11 | Olive/Memeli | Kaş | 2015 | 2 | *Pss* 34 | Olive/Memecik | Antalya | 2015 |
| 1 | *Pss* 14 | Olive/Gemlik | Dösemaltı | 2015 | 2 | *Pss* 38 | Olive/Edremit | Antalya | 2015 |
| 1 | *Pss* 15 | Olive/Gemlik | Antalya | 2015 | 2 | *Pss* 41 | Olive/Kan | Kaş | 2016 |
| 1 | *Pss* 17 | Olive/Kan | Antalya | 2015 | 2 | *Pss* 46 | Olive/Memecik | Kaş | 2016 |
| 1 | *Pss* 18 | Olive/Edinciksu | Antalya | 2015 | 2 | *Pss* 50 | Olive/Gemlik | Kaş | 2016 |
| 1 | *Pss* 27 | Olive/Gemlik | Antalya | 2015 | 2 | *Pss* 54 | Olive/Memeli | Antalya | 2016 |
| 1 | *Pss* 28 | Olive/Gemlik | Kaş | 2015 | 2 | *Pss* 58 | Olive/Edinciksu | Kaş | 2016 |
| 1 | *Pss* 31 | Olive/Gemlik | Dösemaltı | 2015 | 2 | *Pss* 59 | Olive/Edinciksu | Kaş | 2016 |
| 1 | *Pss* 32 | Olive/Edinciksu | Serik | 2015 | 2 | *Pss* 62 | Olive/Ayvalık | Antalya | 2016 |
| 1 | *Pss* 33 | Olive/Memecik | Serik | 2016 | 2 | *Pss* 65 | Olive/Gemlik | Antalya | 2018 |
| 1 | *Pss* 35 | Olive/Gemlik | Serik | 2016 | 2 | *Pss* 67 | Olive/Gemlik | Antalya | 2018 |
| 1 | *Pss* 36 | Olive/Gemlik | Kaş | 2016 | 3 | *Pss* 12 | Olive/Gemlik | Serik | 2015 |
| 1 | *Pss* 37 | Olive/Memeli | Kaş | 2016 | 3 | *Pss* 19 | Olive/Edremit | Aksu | 2015 |
| 1 | *Pss* 39 | Olive/Kan | Aksu | 2016 | 3 | *Pss* 21 | Olive/Memeli | Kaş | 2015 |
| 1 | *Pss* 40 | Olive/Edinciksu | Dösemaltı | 2016 | 3 | *Pss* 23 | Olive/Kan | Aksu | 2015 |
| 1 | *Pss* 42 | Olive/Kan | Dösemaltı | 2016 | 3 | *Pss* 30 | Olive/Gemlik | Serik | 2015 |
| 1 | *Pss* 44 | Olive/Edremit | Dösemaltı | 2016 | 3 | *Pss* 43 | Olive/Gemlik | Serik | 2015 |
| 1 | *Pss* 45 | Olive/Edremit | Serik | 2016 | 3 | *Pss* 49 | Olive/Gemlik | Kaş | 2016 |
| 1 | *Pss* 47 | Olive/Kan | Serik | 2016 | 3 | *Pss* 53 | Olive/Gemlik | Kaş | 2016 |
| 1 | *Pss* 48 | Olive/Gemlik | Kaş | 2016 | 3 | *Pss* 60 | Olive/Kan | Serik | 2016 |
| 1 | *Pss* 51 | Olive/Gemlik | Kaş | 2016 | 3 | *Pss* 61 | Olive/Ayvalık | Dösemaltı | 2016 |
| 1 | *Pss* 52 | Olive/Gemlik | Antalya | 2016 | 3 | *Pss* 66 | Olive/Gemlik | Dösemaltı | 2018 |
| 1 | *Pss* 55 | Olive/Gemlik | Aksu | 2016 | 1 | *Pss*I2 | Olive/Kan | Aksu | 2000 |
| 1 | *Pss* 56 | Olive/Edinciksu | Aksu | 2016 | 1 | *Pss*I7 | Olive/Kan | Serik | 2000 |
| 1 | *Pss* 57 | Olive/Memecik | Kaş | 2016 | 1 | *Pss*I21 | Olive/Kan | Kaş | 2000 |
| 1 | *Pss* 63 | Olive/Memecik | Dösemaltı | 2018 | 4 | *Pss* I24 | Myrtus | Döşemaltı | 2000 |
| 1 | *Pss* 64 | Olive/Kan | Dösemaltı | 2018 | 3 | *Psn* 9 | Nerium | Serik | 2000 |
| 2 | *Pss* 5 | Olive/Kan | Aksu | 2015 | 1 | NCPPB639 | Olive | Yugoslavia | 1908 |
| 2 | *Pss* 8 | Olive/Gemlik | Antalya | 2015 | | | | | |


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### *PFGE*

The *Psv* isolates were cultured in 5 ml Nutrient Broth at 28 °C at 140 rpm shaking for 24 h. Cell suspensions were adjusted to an OD of 0.3 A (approximately $1 \times 10^8$ CFU/ml) at 600 nm by utilizing a spectrophotometer (Eppendorf, Hamburg, Germany). The bacterial suspensions (1.5 ml) were centrifugated at 14,000 rpm for 2 min, pelleted, and resuspended in 1 ml sterile $dH_2O$. This procedure was repeated twice. After suspension of the cells in 500 µl TE buffer (10 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 25 mM EDTA, pH 8.0),$1 \times 10^8$ CFU/ml cells were encapsulated in 500 µl 2% (w/v) low melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA), and transferred into sterile fitting-molds (Bio-Rad Laboratories, Hercules, CA, USA). The hardened agarose fittings at 4 °C for 20 min were returned to a 1.5 ml microfuge tube. Agarose fittings with 1 mg/ml Proteinase K in 250 mM EDTA (pH 9.5) and 25% (w/v) N-laurylsarcosine were incubated overnight at 50 °C. The agarose plugs were returned to a sterile tube containing 1.5 ml sterile 250 mM EDTA (pH 8.0) at 4 °C until used. Complete restriction digestion of 1/5 of agarose fittings was performed with 15 U of *Spe* I, *Ase* I, and *Xba* I (Hacıoğlu et al., 1997) at 37 °C for 7 h. The fittings were inserted into 0.9% (w/v) agarose gel in 0.5x TBE buffer [44.5 mM Tris-HCl, 44.5 mM Boric Acid, 1 mM EDTA (pH 8.0)] at 14 °C all through the kept running by utilizing a pulsed field gel electrophoresis unit (CHEF-DR III) (Bio-Rad Laboratories Hercules, CA, USA).

The optimal migration condition was used, depending on DNA size, at 200 V with an angle of 120°, with an underlying switch time of 5 s and a last switch time of 45 s for 22 h. The gel was stained with 0.5x TBE buffer containing 0.5 µg/ml ethidium bromide for 200 min. Low–Range (New England Biolabs, MA, USA) and Yeast Chromosomal PFGE (*Saccharomyces cerevisiae*) markers were utilized as molecular size markers. The gel image was obtained by using a VilberLourma Monochrome ½" IT CCD camera. DNA fingerprint patterns and sizes were analysed by Bio-gene gel imaging programming (Vilber Lourma, France). UPGMA (Sneath and Sokal, 1973) and Dice coefficient (2a/(2a + b + c) (Dice, 1945) were utilized for the DNA fingerprint matching and dendrogram analyses.

## Results

### *Isolation and identification of bacteria*

The isolates and their locations are given in *Figure 1* and *Table 2*. The pathogens were characterized after isolation from infected olive trees, thereby establishing their presence across the various districts of Antalya province in Turkey. The incidence of the olive knot disease reported for each district by Bansal et al. (1994) are: 80% in Serik, 20% in Döşemealtı-Kırkgöz, 17% in Aksu-Topallı, 10% in Kaş-Dalyan, and 4% in Antalya-Center.

*Psv* grew well and produced unique levan-negative colonies on selective PVF-1, KB, and NSA media. The colonies grown on KB medium were flat, with a diameter ranging from 1–3 mm, greyish to white colour, and irregular margins. The bacteria produced fluorescence pigment on both KB and PVF-1 media under UV light. The colonies grown on PVF-1 were greyish white, slightly raised, smooth, and relatively small (2–3 mm). The colonies grown on NSA medium were grey or pale yellow, slightly raised or flat with a diameter of 3–5 mm. LOPAT indicated negative levan, oxidase, potato


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soft rot, and arginine dehydrolase tests, but all isolates showed positive hypersensitive reaction in the tobacco plant. In all, 67 isolates were recovered from diseased olive trees in Turkey and identified by PCR. The numbers of groups and strains in each group are presented in *Table 2*.

The pathogenicity of the isolates on the one-year-old Gemlik olive seedlings produced characteristic knot symptoms with variable sizes. The healthy olive seedling treated with sterile $dH_2O$ as a control did not show any symptoms (*Fig. 2a*). The pathogen was reliably re-isolated from the knots in the repeated tests, establishing *Psv* as the causal agent of the knot or gall disease in olive plants, thereby satisfying Koch's postulates (*Fig. 2b* and *c*).

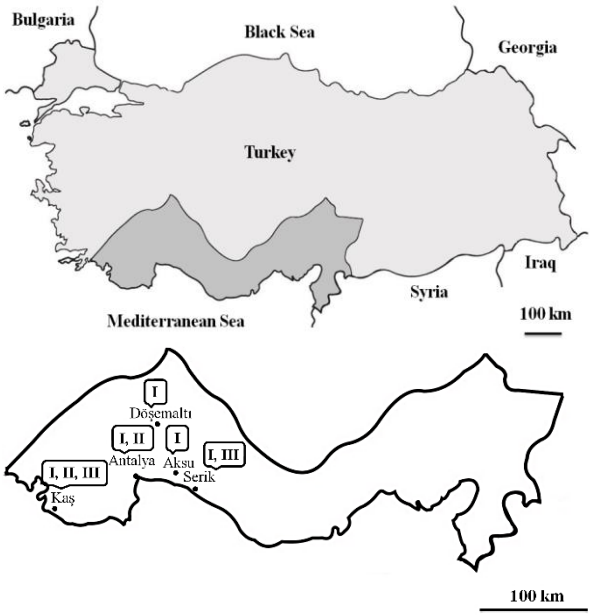


***Figure 1.*** *Distribution of Psv in the western Mediterranean region of Turkey as determined by PFGE analysis. Schematic map of the region showing the 5 districts studied. Letters next to the district names indicate the haplotype groups (I–III)*

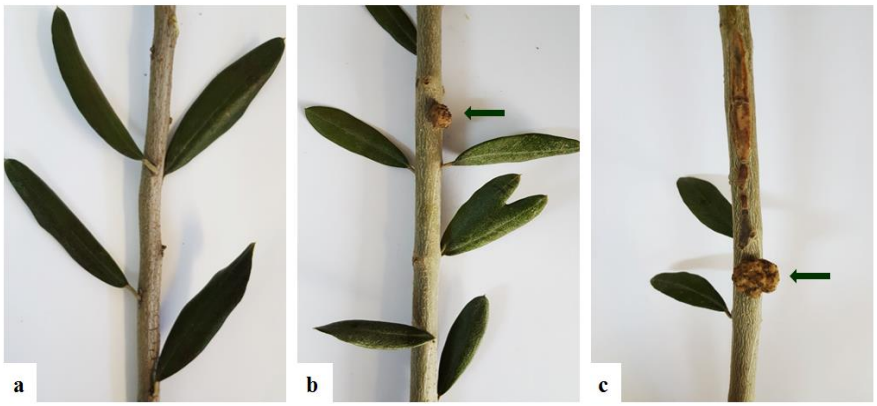


***Figure 2.*** *The pathogenicity of the isolates on a one-year-old Gemlik cultivar of olive seedlings; (a) healthy olive seedling treated with sterile $dH_2O$ used as a control, (b) seedling inoculated with the reference strain (NCPPB 639) showing knot symptoms, (c) seedling inoculated with the Turkish isolate (Pss 2) showing knot symptoms*


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The relationships among the *Psv* strains were established based on FAME analysis. All 67 isolates recovered from the olive plant hosts in Antalya province and the reference strains were analysed. The results show percent similarities of 78.50–100% to the MIS library. The major fatty acids were palmitic acid (16:0), palmitoleic acid (16:1), and oleic acid (18:1) (*Table 3*). The FAME cluster analysis based on the Euclidean distance categorized the Turkish olive strains into 2 groups (*Fig. 3*). Each clade is unique, showing a close relationship among each other.

***Table 3.*** *The percentage values* of total fatty acid methyl esters (FAME) of Psv and Psn isolates*

| FAME | Strain groups | | | |
|---|---|---|---|---|
| | I | II | III | *Psn* 9 |
| 10:0 | 0.0 ± 0.07 | 0.0 ± 0.02 | 0.17 ± 0.03 | 0.0 ± 0.01 |
| 10:0 3OH | 4.50 ± 1.20 | 4.38 ± 1.04 | 2.66 ± 0.24 | 4.28 ± 0.11 |
| 12:00 | 2.00 ± 0.31 | 6.07 ± 0.52 | 4.69 ± 0.32 | 2.59 ± 0.04 |
| 11:00 iso 3HO | 0.0 ± 0.05 | 0.0 ± 0.06 | 0.35 ± 0.05 | 0.0 ± 0.01 |
| 12:0 2OH | 6.41 ± 0.32 | 4.40 ± 0.01 | 3.40 ± 0.92 | 5.88 ± 0.68 |
| 12:0 3OH | 4.70 ± 1.21 | 5.27 ± 1.12 | 4.19 ± 0.59 | 4.21 ± 0.67 |
| 14:0 | 0.42 ± 0.03 | 0.0 ± 0.01 | 0.18 ± 0.05 | 0.43 ± 0.07 |
| 16:1[a] | 34.60 ± 2.58 | 33.45 ± 2.31 | 22.98 ± 2.73 | 35.14 ± 2.99 |
| 16:0[b] | 27.01 ± 1.47 | 20.72 ± 1.78 | 23.19 ± 1.62 | 26.53 ± 1.25 |
| 17:0 iso | 0.0 ± 0.04 | 0.0 ± 0.02 | 1.61 ± 0.03 | 0.0 ± 0.01 |
| 17:0 cyclo | 3.15 ± 0.34 | 0.0 ± 0.05 | 4.42 ± 0.37 | 2.87 ± 0.46 |
| 18:1[c] | 15.34 ± 3.05 | 23.02 ± 2.04 | 27.40 ± 2.81 | 16.49 ± 3.01 |
| 18:0 | 1.09 ± 0.54 | 1.53 ± 0.03 | 2.80 ± 0.89 | 1.57 ± 0.74 |
| 18:1 w7c 11-methyl | 0.0 ± 0.03 | 0.0 ± 0.01 | 1.13 ± 0.02 | 0.0 ± 0.05 |
| Sum In Feature 7 | 0.0 ± 0.40 | 1.15 ± 0.20 | 0.37 ± 0.11 | 0.0 ± 0.02 |
| 19:0 cyclo w8c | 0.0 ± 0.04 | 0.0 ± 0.01 | 0.46 ± 0.02 | 0.0 ± 0.01 |

*Each value is an average ± standard deviation of strain groups
I, II: Olive strain groups of *Psv*., III: *Myrtus communis* strain group of *Psv*
[a]Palmitoleic acid, [b]Palmitic acid, [c]Oleic acid

### *Rep-PCR*

The primer, BOXA1R, which was used to amplify the repetitive DNA sequence of *Psv*, showed various genomic fingerprints. The results obtained by the BOXA1R primer pair showed variability among the *Psv* strains. This primer pair produced different polymorphic patterns among the strains with amplification fragments ranging from ~250 to 2250 bp (*Fig. 4*). BOX PCR cluster analysis categorized all the Turkish olive strains into 2 groups (*Fig. 5*).

### *PFGE*

All 67 isolates and reference strains were evaluated by using PFGE. The results showed various discrete DNA patterns of *Psv* after digesting the total genome with *Spe*I. The restriction digestion of the *Psv* genome yielded fragments which ranged from 9 to 1000 kb (*Fig. 6*). *Ase* I and *Xba* I did not effectively digest genomic DNA in this study. The Turkish olive strains were separated into 3 discrete PFGE groups (*Fig. 7*). Based on the cluster analysis, there was nearly identical linkage between the haplotype


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group I and the reference strain. The percentage similarity among the haplotypes as shown by the cluster was 42–100% (*Fig. 7*). Based on the cluster analysis of PFGE, haplotype group I consisted of 38 haplotypes and reference strain NCPPB639; haplotype group II (21 haplotypes): haplotype group III (11 haplotypes), and the myrtus isolate was placed into haplotype group IV as *Figure 7* and *Table 2.*

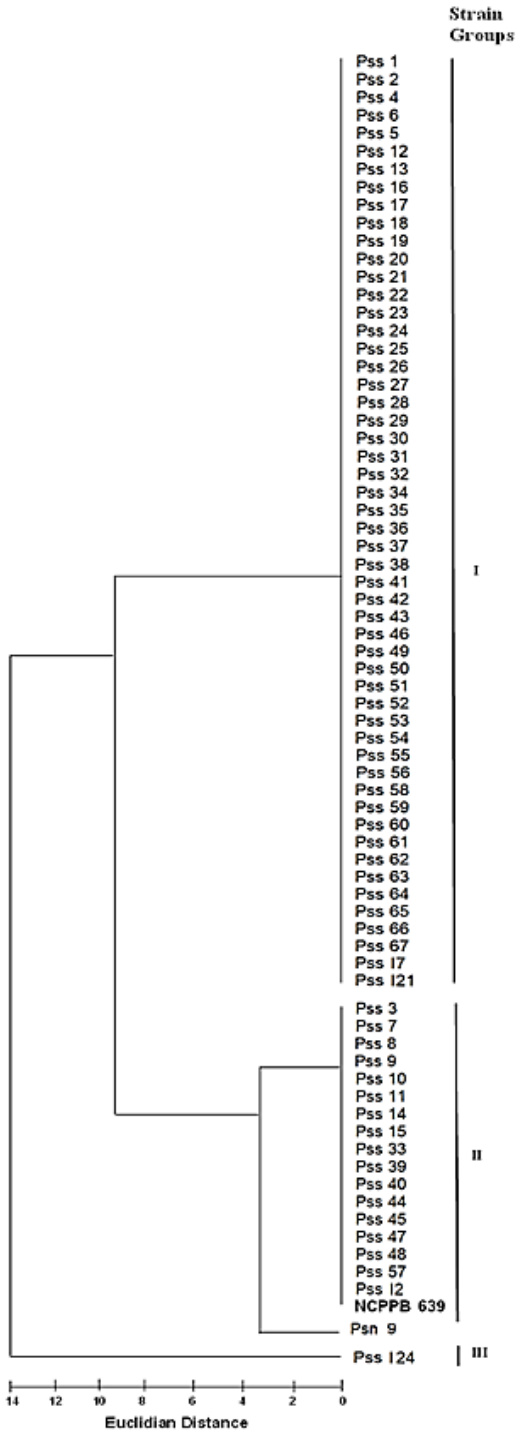


***Figure 3.*** *Dendrogram generated by cluster analysis of Psv strains (Table 2) based on fatty acid composition*


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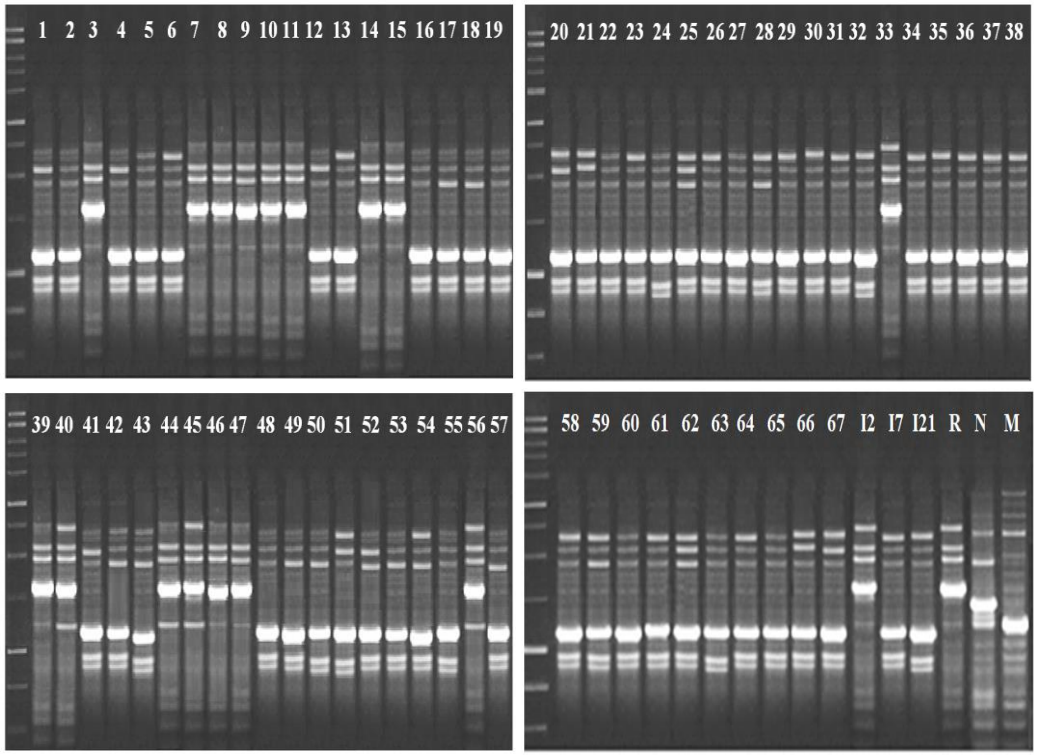


***Figure 4.*** *Agarose gel electrophoresis of repetitive sequence-based BOX-PCR fingerprint patterns obtained from Psv and Psn strains. Lanes: 1–67; Pss 1 – Pss 67, respectively; Lane: I2, I7, I21, R, N, M; Pss I2, Pss I7, Pss I21, NCPPB 639, Psn 9, Pss I24, respectively*

## Discussion

Of the isolates collected from diseased olive plants during the 2015–2018 survey, 67 were found to be heterogenic and divided into 3 haplotype groups by PFGE. From the survey results, the highest percentage of the disease and the olive plant cultivars from which the pathogen were isolated were from the Serik district, and the highest infected (80%) or susceptible cultivar from this district was the Gemlik cultivar. The least infected orchard recorded was from the Antalya-center district. When all the collected samples across the various districts were compared, the Gemlik cultivar showed the highest percentage of infection. Some commercial olive cultivars have been found to be considerably tolerant to olive knot disease (Penyalver et al., 2006). The high rate of infection in the Gemlik cultivar could be due to a high susceptibility to this pathogen. It could also be also be attributed to the exposure of these varieties to powerful winds common in the coastal regions of Antalya-center and Kaş-Dalyan. These strong winds create wounds on the plants which allow entrance for the pathogen. Additionally, the fungus, *Cycloconium oleaginum*, a causal agent of olive leaf spot disease that results in defoliation, creates wounds that can serve as entrance point for *Psv*.

The different groups of *Psv* strains according to PFGE occurred in the same districts because of the large olive plant production as well as different cultivars of olive grown in these areas. There was no association of genetic diversity of Turkish *Psv* population with olive cultivars of isolation. The results of Scortichini et al. (2004) were supported by our findings. On the other hand, Moretti et al. (2008) and Krid et al. (2009) reported an association with geography and olive cultivars.


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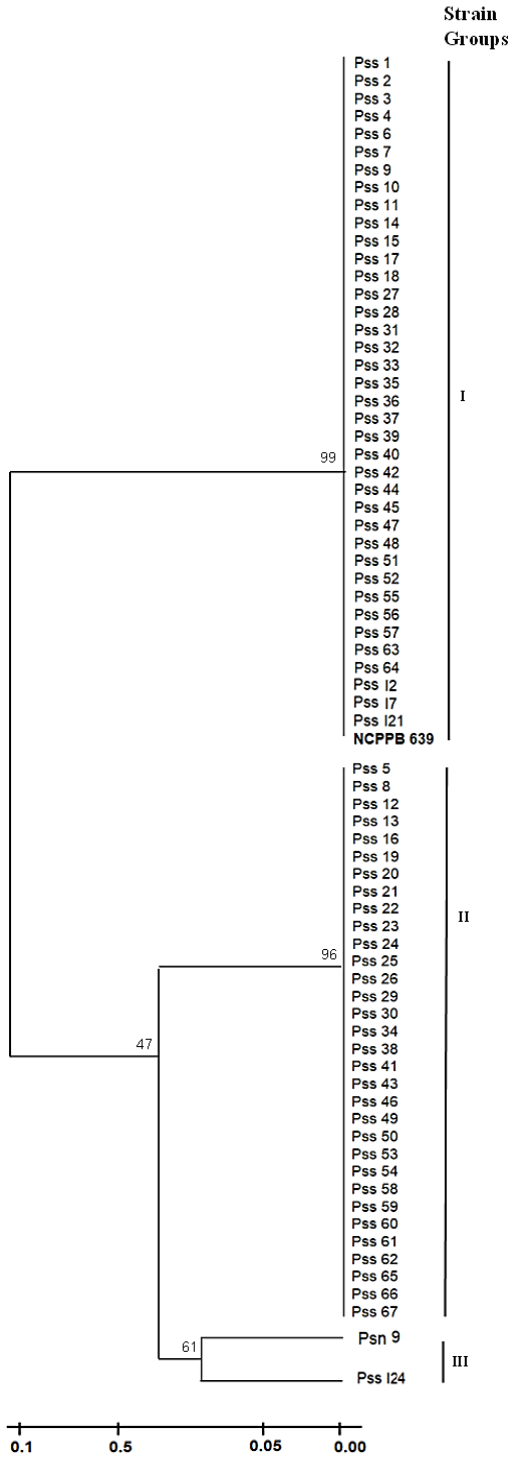


***Figure 5.*** *Unweighted average linkage dendrogram of the cluster analysis of Psv strains (Table 2) based on BOX-PCR analysis*

Understanding the *Psv* population may help to enhance the present control management of olive knot disease. *Psv* isolates obtained from infected olive trees in various geographical region of the world are different. In a few studies, various *Psv* populations were gathered from various parts of the world, including 360 isolates from


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Italy (Scortichini et al., 2004), 71 isolates from Italy, USA, Greece, Portugal, former Yugoslavia, UK, the Netherlands, and France (Sisto et al., 2007), 62 isolates from Spain, Italy, Serbia, France, Portugal, USA, Algeria, Greece, Jordan, and Tunusia (Quesada et al., 2008), 58 strains from Tunusia (Krid et al., 2009), 31 isolates from Italy (Cinelli et al., 2014), 124 isolates from various Mediterranean countries (Moretti et al., 2017), and 19 isolates from Japan (Tsuji et al., 2017)

Scortichini et al. (2004) analysed 360 Italian *Psv* isolates and reported the presence of 20 DNA fingerprint patterns by rep-PCR. The 44 Spanish *Psv* isolates were grouped into 3 different groups, and the only 6 Italian isolates were placed into 3 different groups by the presence of IS*53* (Quesada et al., 2008). The 58 Tunisian *Psv* isolates were clustered into 3 different groups by RAPD (Krid et al., 2009). Recently, Moretti et al. (2017) analysed 124 *Psv* isolates from 15 different countries and grouped the isolates into 2 clusters and 4 subclusters by MLST and rep-PCR. The 19 Japanese isolates were differentiated into 5 different groups by rep-PCR (Tsuji et al., 2017).

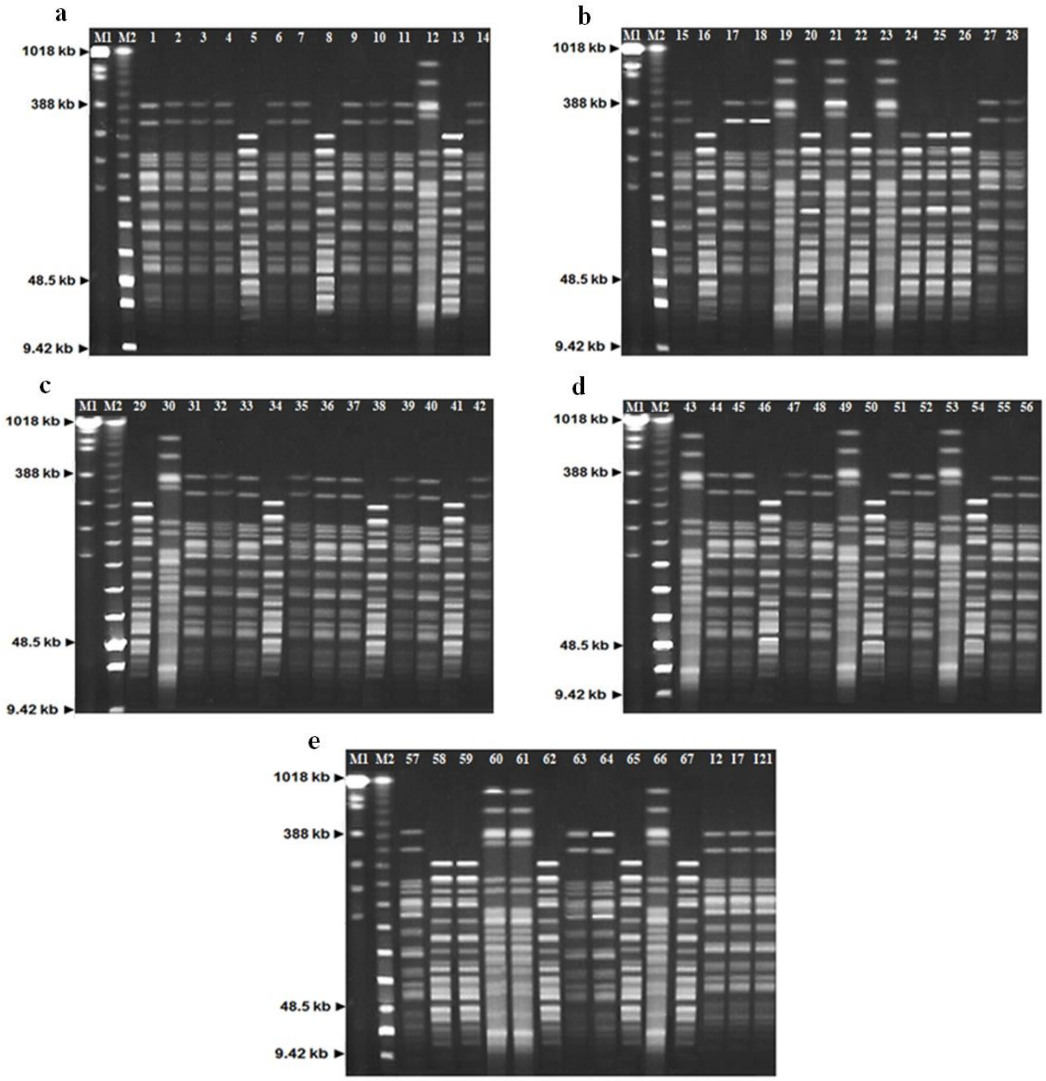


***Figure 6.*** *(a, b, c, d, e) Genomic DNA fingerprints from olive strains of Psv in Turkey by PFGE using Spe I. Lanes: M1 = DNA size standards of Saccharomyces cerevisiae, M2 = Low-range PFGE marker. Lanes: 1–67 = Pss1–Pss67, respectively; Lane: I2, I7, I21 = PssI2, PssI7, PssI21, respectively*


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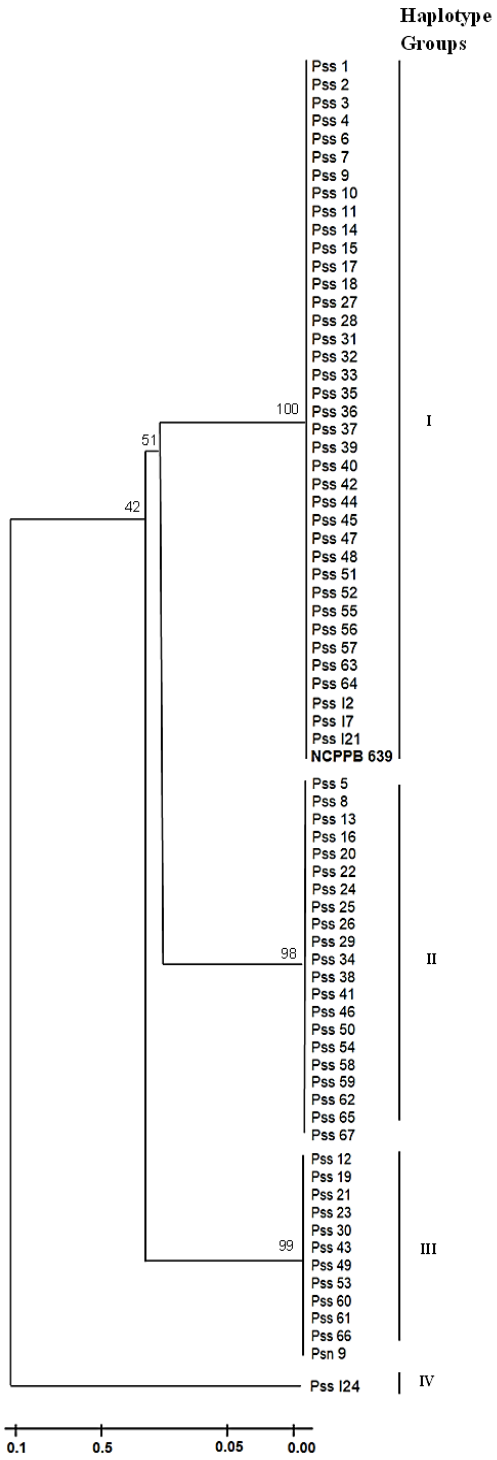


***Figure 7.*** *Unweighted average linkage dendrogram of cluster analysis of Psv (Table 2) haplotype groups generated by PFGE patterns with Spe I. Number of horizontal axes indicates percent similarity as determined by Dice coefficient*

There have been reports of the presence of olive knot disease caused by *Psv* in the Aegean region, which is one of the olive growing provinces of Turkey (Tatlı and Benlioğlu, 2004) and in the western Mediterranean region, specifically in Antalya


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province (Basım and Ersoy, 2000), in oleander plants in the South-eastern Anatolia, specifically in Şanlıurfa (Kavak and Üstün, 2009), and from the Adana, Samsun, and Tekirdağ provinces of Turkey (Mirik et al., 2011).

The morphological and biochemical tests indicated that the 3 media used in this study produced colonies typical of *Psv*. Considerable heterogeneity in the colonies established by *Psv* strains isolated from different cultivars of olive in various localities have been reported in Italy (Surico and Marchi 2003). LOPAT showed isolates to be levan negative, and all isolates produced a positive hypersensitive reaction in tobacco plant. The LOPAT profile (Lelliott et al., 1966) and other tests described by Lelliott and Stead (1987) were used to separate a typical *Psv* strain from *P. syringae* subsp. *syringae*. Janse (1981) observed that some *Psv* strains isolated from various host plants had almost similar physiological and biochemical dispositions. All isolates were confirmed to be pathogenic on a one-year-old olive plant, but not on an oleander plant. Our result is in concordance with the findings reported by Perez-Martinez et al. (2007).

*Table 3* indicates the relative (percent) total fatty acid methyl esters as found by FAME analysis of the *Psv* isolates, which were entered into the standard MIDI library. The FAME cluster categorized all the strains isolated from olive plants collected from various districts of Antalya province which were clustered into 2 groups based on Euclidean metrics. The FAME analysis provides useful information with regards to the phenotypic identification of the pathogen, but it is not an effective technique for discriminating between strains.

The diversity study was carried out using rep-PCR with primer pair, BOXA1R, which was found to be informative and discriminatory for all the *Psv* isolates tested in this study, and produced polymorphic patterns corresponding to different amplification fragment sizes (*Fig. 4*). All the strains isolated from olive plants formed 2 groups based on the BOX-PCR. Although the BOX-PCR was found to be effective at differentiating *Psv* isolates, its discriminating ability was less than that of PFGE as observed in this study. Interestingly, it could be seen from the cluster tree that strains from different districts of a province or geographical location showed similar genetic homogeneity and were grouped together in one clade by the BOX-PCR cluster analysis (*Fig. 5*).

The genetic variability among the *Psv* strains was further confirmed by using PFGE after restriction digestion with *Spe* I, a rare-cutting enzyme. Three different haplotype groups were produced based on the DNA fragment patterns generated as compared to FAME and BOX-PCR analyses, which categorized the isolates from olive plant hosts into 2 groups each. The results of this study recommend that the FAME and BOX-PCR strategies were not capable as PFGE to separate *Psv* strains. The PFGE utilizing rare-cutting endonuclease *Spe* I given the foremost comprehensive comes about. The effective discriminatory ability and reproducibility of PFGE makes it one of the foremost broadly utilizied method for comparative fingerprinting of most bacterial species (Sails et al., 2003; Lukinmaa et al., 2004; van Belkum et al., 2007). Based on PFGE, most *Psv* strains were placed in haplotype group I with reference strain NCPPB639. The other strains were separated into 2 haplotype groups, resulting in a total 3 different haplotype groups in the olive *Psv* population. This heterogeneity among the *Psv* population can be clarified by even exchange of plasmids and chromosomal genes as seen in *Xanthomonas* and *Pseudomonas* pathovars (Canteros et al., 1995; Basım et al., 1999; Sundin, 2007). Based on PFGE, the isolate *Pss*9 from *Nerium oleander* was placed in haplotype group II, along with several olive strains. However, the same strain was placed in a different haplotype group by rep-PCR analysis.


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Comparative comes about were found by Moretti et al. (2017). *P. s*. pv. *nerii* and *P. s.* pv. *fraxinii* were plainly isolated from the *Psv* population by utilizing rep-PCR. Based on MLST, isolates of *P. s.* pv. *nerii* and *P. s.* pv. *fraxini* pathovars clearly have a same genetic feature as *Psv*, and may have adjusted to oleander and fraxinus, individually, after *Psv* risen as an olive plant pathogen (Moretti et al., 2017).

## Conclusion

Our results show that PFGE using *Spe*I has an effective discriminatory capability for genotypic analysis of *Psv* population. The present study provides important outputs to better comprehension of the genotypic and phenotypic structure of the *Psv* population in Turkey. The results about given an incredible opportunity for following strain shifting in the *Psv* population in future, and for olive breeding programs pointed at the advancement of an olive cultivar safe to the distinctive *Psv* strains.

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