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Research Article

# Variation in extragenic repetitive DNA sequences in *Pseudomonas syringae* and potential use of modified REP primers in the identification of closely related isolates

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## Abstract

In this study, *Pseudomonas syringe* pathovars isolated from olive, tomato and bean were identified by species-specific PCR and their genetic diversity was assessed by repetitive extragenic palindromic (REP)-PCR. Reverse universal primers for REP-PCR were designed by using the bases of A, T, G or C at the positions of 1, 4 and 11 to identify additional polymorphism in the banding patterns. Binding of the primers to different annealing sites in the genome revealed additional fingerprint patterns in eight isolates of *P. savastanoi* pv. *savastanoi* and two isolates of *P. syringae* pv. *tomato*. The use of four different bases in the primer sequences did not affect the PCR reproducibility and was very efficient in revealing intra-pathovar diversity, particularly in *P. savastanoi* pv. *savastanoi*. At the pathovar level, the primer BOX1AR yielded shared fragments, in addition to five bands that discriminated among the pathovars *P. syringae* pv. *phaseolicola*, *P. savastanoi* pv. *savastanoi* and *P. syringae* pv. *tomato*. REP-PCR with a modified primer containing C produced identical bands among the isolates in a pathovar but separated three pathovars more distinctly than four other primers. Although REP- and BOX-PCRs have been successfully used in the molecular identification of *Pseudomonas* isolates from Turkish flora, a PCR based on inter-enterobacterial repetitive intergenic concensus (ERIC) sequences failed to produce clear banding patterns in this study.

*Key words:* bacterial identification, biodiversity, PCR.

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## Introduction

The *Pseudomonas syringae* group belongs to the family Pseudomonaceae and causes diseases in nearly all species of cultivated plants, including horticultural crops and ornamental and fruit trees (Young *et al.*, 1996). Many of these pathovars, which are widely distributed on different plant parts (shoots, leaves, buds, pots, etc.), are highly specific to one or a few related plant species and cause a variety of symptoms such as watersoaking, hypertrophic growth, cankers, chlorosis and necrosis (Murillo and Sesma, 2001). The *Pseudomonas syringae* group also has the best described epiphytic growth among phytopathogenic bacteria (Hirano and Upper, 2000). One member of this group, *Pseudomonas savastanoi*, has been classified as a species rather than a pathovar based on DNA hybridization and ribotyping studies (Gardan *et al.*, 1999). Pathogenic strains of *P. savastanoi* pv. *savastanoi* infect olive trees and cause olive knot disease.

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*Pseudomonas syringae* has previously been identified based on fatty acid profiling (Stead, 1992), protein analyses (Van Zyl and Steyn, 1990) and plasmid profiles (King, 1989). More recently, DNA fingerprinting methods such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) (Cirvilleri *et al.*, 2006; Sisto *et al.*, 2007), restriction fragment length polymorphism (RFLP) (Manceau and Horvais, 1997) and insertion sequence-based methods (Weingart and Völksch, 1997; Oguiza *et al.*, 2004; Quesada *et al.*, 2008) have been used to diagnose and genotype *P. syringae* strains and pathovars of different origins. However, none of these methods is ideal for such identifications since each of them has technical disadvantages. PCR-based methods are still the most preferred approach for bacterial genotyping because their low-cost and high output make them efficient in providing new genomic sequence information. Bacterial extragenic non-coding regions have been widely used to obtain specific fingerprints that are more informative and reproducible than RAPD markers.

Repetitive extragenic palindromic (REP) sequences or elements were first described in *Escherichia coli* and

*Salmonella typhimurium* operons (Higgins *et al.*, 1982). The palindromic nature of these elements and their ability to form stable stem-loop structures in transcribed RNA suggest that they may have regulatory roles associated with transcriptional termination, mRNA stability and chromosomal organization in bacteria (Versalovic *et al.*, 1991). Amplifications based on REP elements in conjunction with other related families of repetitive elements, such as enterobacterial repetitive intergenic concensus (ERIC) and BOX sequences, have been exploited in the molecular identification of bacteria pathogenic to plants. Three types of PCR (known as Rep-PCR) based on these elements have been used together and specific reproducible fingerprints were obtained more quickly and more cost-effectively than with other methods such as AFLP and RFLP (Louws *et al.*, 1999).

Rep-PCR genotyping has been applied to many species of plant pathogenic bacteria but has limited ability in discriminating among pathovars and closely related isolates (de Bruijn, 1992; Judd *et al.*, 1993; Woods *et al.*, 1993). One reason for this poor discriminatory power is that REP-PCR may produce monomorphic bands in bacterial samples collected from distant geographic locations. Another reason is the variability in the PCR amplification products obtained for the three types of repetitive elements in bacterial genomes. The universal primers of REP-PCR amplify the intervening DNA between two adjacent repetitive elements. We have modified reverse REP-PCR primers produced by substituting A, T, G or C at three different positions to yield four primer combinations. By changing the annealing sites in the genome we obtained highly polymorphic fingerprint patterns among *P. savastanoi* pv. *savastanoi* isolates collected from olive trees. In the present study, we used a set of these primers with high discriminatory power to distinguish isolates and pathovars of *P. savastanoi* pv. *savastanoi*, *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato*.

## Materials and Methods

### Bacterial strains

*Pseudomonas syringae* pathovars of tomato, bean and olive (Pss-14) (Table 1) were kindly provided by Prof. Hatice Ozaktan (Ege University), Dr. Aynur Karahan (Ankara Plant Protection Central Research Institute) and Prof. Kemal Benlioglu (Adnan Menderes University), respectively. All of the pathovars originated in Turkey except for NCPPB-52, which originated in the United Kingdom (UK). *Pseudomonas savastanoi* pv. *savastanoi* isolates other than Pss-14 were isolated from olive knots of branches obtained from nurseries in the Marmara and Aegean regions (western and northwestern Turkey).

**Table 1** - *Pseudomonas savastanoi* and *P. syringae* isolates used in this study.

| Pathovar | Strain | Isolate | Host | Location |
|---|---|---|---|---|
| *Pseudomonas savastanoi* | pv. *savastanoi* | Pss-14 | Olive | Antalya |
| | pv. *savastanoi* | Pss-7A | Olive | Orhangazi |
| | pv. *savastanoi* | Pss-8A | Olive | Orhangazi |
| | pv. *savastanoi* | Pss-9A | Olive | Orhangazi |
| | pv. *savastanoi* | Pss-7D | Olive | Orhangazi |
| | pv. *savastanoi* | Pss-4B | Olive | Orhangazi |
| | pv. *savastanoi* | Pss-M9 | Olive | Akhisar |
| | pv. *savastanoi* | Pss-M25 | Olive | Akhisar |
| *Pseudomonas syringae* | pv. *tomato* | Pst | Tomato | Izmir |
| | pv. *tomato* | Pst-101 | Tomato | Ankara |
| | pv. *phaseolicola* | Psp-3 | Bean | Ankara |
| | pv. *phaseolicola* | Psp-18 | Bean | Ankara |
| | pv. *phaseolicola* | Psp-R52 | Bean | Izmir |
| | pv. *phaseolicola* | NCPPB-52 | Bean | UK |

### Isolation and identification of *P. savastanoi* pv. *savastanoi*

*P. savastanoi* pv. *savastanoi* was isolated as described by Saygili (1995). Briefly, knots were washed in tap water and cut into small fragments (2 x 2 mm) with a sterile scalpel. The fragments were placed in sterile tubes containing 3 mL of sterile distilled water and left at room temperature for 30 min. Subsequently, ~20 µL aliquots were streaked on King B (KB) medium (King *et al.*, 1954) in petri dishes and incubated at 28 °C for 72 h. The suspected colonies of *P. savastanoi* pv. *savastanoi*, which were flat and 2-3 mm in diameter with irregular margins and a grayish-white color, were selected and spread again on selective PVF-1 medium (Kado and Heskett, 1970) followed by incubation at 28 °C for 72 h. This subculturing was repeated 2-3 times to purify the isolates. The isolates were also Gram stained. All of the *P. syringae* strains identified (Table 1) were cultured in appropriate medium and stored at -80 °C.

For species-specific PCR and repetitive-PCR, a single colony of each strain of *P. syringae* was used as a source of template DNA. Primer sequences (Pss1: 5'-TGGGTTGCTACTTGTACCGGA-3' and Pss2: 5'-CCGTGTACTACGTTCAGCGAG-3') corresponding to the *ptz* (Basim and Ersoy, 2001) and *iaaL* (IAALF: 5'-GGCACCAGCGGCAACATCAA-3' and IAALR: 5'-CGCCCTCGGAACTGCCATAC-3') genes (Penyalver *et al.*, 2000) were used for *P. savastanoi* pv. *savastanoi*. We also used primers derived from the *argK* (Psp1: 5'-CCATGAAGATTACAAGCCTG-3' and Psp2: 5'-GCTAGCTATCAGGGGACGAC-3') (Mosqueda-Cano and Herrera-Esterella, 1997) and *cfl* (Pst1: 5'-GGCGCTCCCTCGCACTT-3' and Pst2: 5'-GGTATTGGCGGGGGTGC-3') (Bereswill *et al.*,

1994) genes for *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *tomato*, respectively.

The reaction mixture consisted of 1x Ex-*Taq* DNA polymerase buffer (Takara), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 units of Ex-*Taq* DNA polymerase (Takara) and 50 pmol of each primer in a final volume of 25 μL. The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Three independent amplifications were done for each sample in a Techne thermocycler and the PCR products were separated on 1% agarose gels and photographed under UV illumination.

### Repetitive-PCR

Universal primers based on REP sequences were used for REP-PCR (Versalovic *et al.*, 1991). The REP primers were modified by inserting a base (A, T, G or C) at one of three N positions in the reverse Rep-2-Dt primer (Table 2). We examined five primer sets that included different combinations of REP primers (Rep-1R-Dt/Rep-2-Dt, Rep-1R-Dt/Rep-2A, Rep-1R-Dt/Rep-2T, Rep-1R-Dt/Rep-2G and Rep-1R-Dt/Rep-2C). The primer used for BOX-PCR was BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic *et al.*, 1994) while those used for ERIC-PCR were ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Versalovic *et al.*, 1991).

The reaction mixture consisted of 1x Ex-*Taq* DNA polymerase buffer (Takara), 3 mM $MgCl_2$, 200 μM of each dNTP, 1 unit of Ex-*Taq* DNA polymerase (Takara) and 50 pmol of each primer in a final volume of 25 μL. The PCR conditions included an initial denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for 1 min, 34 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 15 min. Two independent amplifications were done for each sample in a Creon T-cy thermocycler and the PCR products were separated on 1.5% agarose gels and photographed under UV illumination.

### Data analysis

The bands for each strain and primer were scored as absent (0) or present (1) and the resulting fingerprints were compared using MVSP 3.2 software. Jaccard's coefficient of similarity index (Jaccard, 1908) was used to calculate similarity distances. Cluster analysis was done using the unweighted pair-group method with arithmetic average (UPGMA).

**Table 2** - Universal and modified primers used for REP-PCR in this study.

| Primer | Primer sequence (5'-3') |
|---|---|
| Rep-1R-Dt | IIINCGNCGNCATCNGGC (forward) |
| Rep-2-Dt | NCGNCTTATCNGGCCTAC |
| Rep-2A | **A**CG**A**CTTATC**A**GGCCTAC |
| Rep-2T | **T**CG**T**CTTATC**T**GGCCTAC |
| Rep-2G | **G**CG**G**CTTATC**G**GGCCTAC |
| Rep-2C | **C**CG**C**CTTATC**C**GGCCTAC |

Letters in bold type indicate the bases that were altered in each primer at the positions indicated by N (underlined) in the first primer.

## Results

Seven *P. savastanoi* pv. *savastanoi* isolates were obtained from 110 olive knots after elimination of suspected colonies. Isolates 7A and 7D (Table 1) were closely related samples obtained from two knots on the same branch. The bacteria were Gram-negative and grew well on selective PVF-1 medium. All of the isolates produced the expected bands of 684 bp and 454 bp with the primer pairs Pss1/Pss2 and IAALF/IAALR, respectively (Figure 1A,B). Bands of 650 bp and 1000 bp were amplified in all *P. syringae* pv. *tomato* and *P. syringae* pv. *phaseolicola* strains with primer pairs Pst1/Pst2 and 62a/63a, respectively (data not shown).

*P. savastanoi* pv. *savastanoi* genomic fingerprints were obtained with all of the five REP-PCR primer sets. Three primer sets (Rep-1R-Dt/Rep-2-Dt, Rep-1R-Dt/Rep-2A and Rep-1R-Dt/Rep-2C) were more polymorphic among the isolates than the other two sets (Rep-1R-Dt/Rep-2T and Rep-1R-Dt/Rep-2G) (Figure 2). The corresponding PCR products ranged in size from 200 bp to 3000 bp (Figure 2). In two *P. syringae* pv. *tomato* strains from Ankara and Izmir, the REP primer sets produced very distinctive fingerprints, particularly with primer pairs Rep-1R-Dt/Rep-2-Dt, Rep-1R-Dt/Rep-2G and Rep-1R-Dt/Rep-2C (Figure 3). The corresponding PCR products ranged in size from ~200 bp to > 1500 bp.

At the pathovar level, each REP primer set was screened for well-defined, reproducible bands. The Rep1R-Dt and Rep-2C primer pairs distinguished among pathovars, as shown in Figure 4A. Amplification with the BOXA1R primer yielded PCR products of 270-2100 bp in each pathovar (Figure 4B). The arrows in Figure 4B indicate polymorphic bands for tomato, olive and bean patho-

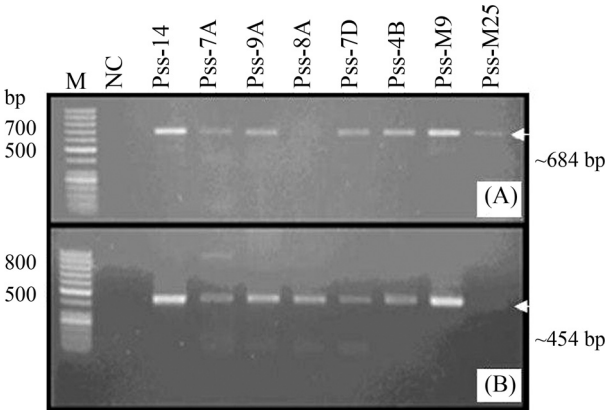


**Figure 1** - Species-specific PCR fragments of *P. savastanoi* pv. *savastanoi* isolates obtained with (A) Pss1/Pss2 and (B) IAALF/IAALR primers. M - DNA ladder, NC - negative control (no DNA template).

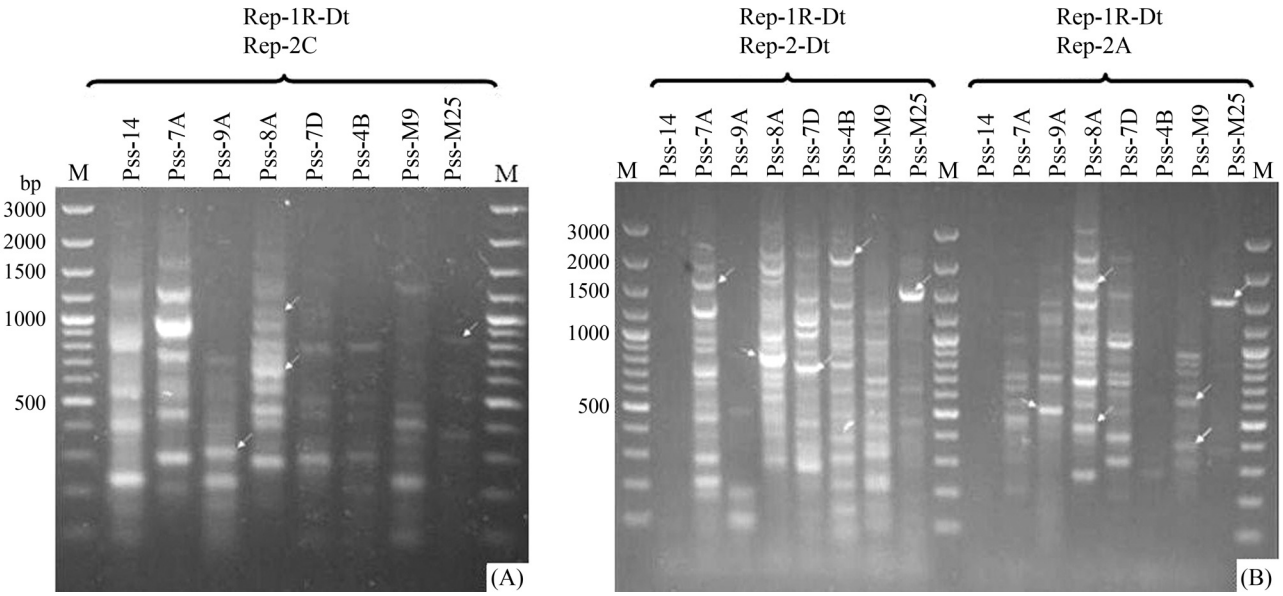


**Figure 2** - Repetitive-PCR fragments of *P. savastanoi* pv. *savastanoi* isolates obtained with different REP primer combinations. (A) Rep-1R-Dt and Rep-2C, (B) Rep-1R-Dt and Rep-2-Dt, and Rep-1R-Dt and Rep-2A. M - DNA ladder.

vars of *P. syringae* (Psp-18, Pss-14 and Pst-101, respectively). REP-PCR yielded a higher number of bands and more complex patterns than BOX-PCR. *P. syringae* pv. *phaseolicola* isolates were distinguishable from all the other isolates by the size of their PCR products (200-700 bp; lanes 1-4 in Figure 4B). Exceptions included the *P. syringae* pv. *phaselicola* pathovar from Ankara (Psp-3), which lacked the 700 bp band, and Psp-18, which produced two additional bands of ~2100 bp and 1500 bp (lane 4 in Figure 4B). *P. savastanoi* pv. *savastanoi* could be distinguished by a single specific fragment of 700 bp (lane 5 in Figure 4B). *P. syringae* pv. *tomato* strains differed completely from the other pathovars and from each other by producing two slightly different bands of ~250 bp (lanes 6 and 7 in Figure 4B). In contrast to REP-PCR which produced several common bands in tomato pathovars, no common bands were observed with BOX-PCR (lanes 6 and 7 in Figure 4B). The monomorphic and polymorphic bands observed with BOXA1R in *P. syringae* pv. *phaselicola* isolates of Turkish and UK origin demonstrated the conserved nature of BOX elements in the *P. syringae* genome. No observable inter-ERIC fingerprints were obtained in six local isolates or in isolate NCPPB-52 of UK origin.

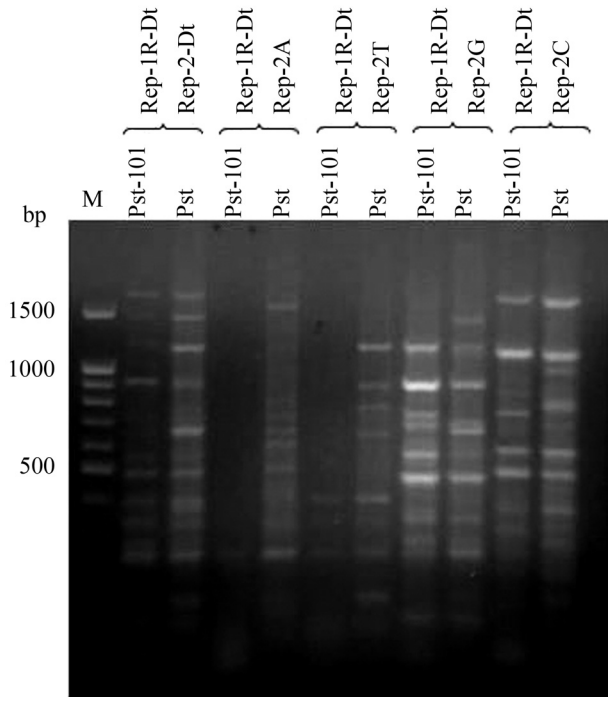


**Figure 3** - Repetitive-PCR fragments of *P. syringae* pv. *tomato* isolates obtained with different REP primer combinations. M - DNA ladder.

The UPGMA dendrogram revealed two major divisions among the isolates (Figure 5) the *P. syringae* pv. *phaseolicola* pathovars formed a group that included *P. savastanoi* while the *P. syringae* pv. *tomato* isolates formed a second group, with a genetic similarity of 0.25 between groups. The approximate genetic diversity among all isolates was 80%.

## Discussion

The precise identification of *P. syringae* pathovars, which are widespread pathogens, is important for basic studies related to genetic polymorphism and adequate agricultural management. Species-specific PCR is a powerful method for the rapid, convenient diagnosis of microbial pathogens in laboratory samples derived from plants with symptomatic phenotypes. The Pss1/Pss2 primer pair used by Basim and Ersoy (2001) for the molecular identification

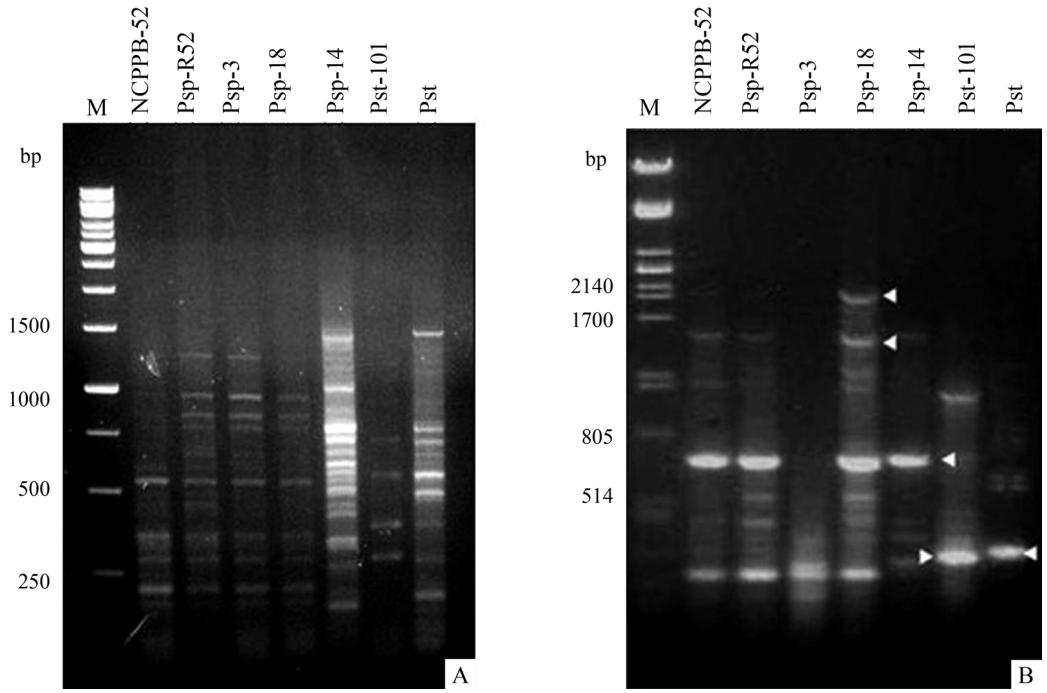


**Figure 4** - REP-PCR fingerprints of *P. syringae* pathovars obtained with Rep-1R-Dt and Rep-2C primers (A) and BOX-PCR fingerprints of *P. syringae* pathovars (B). M - 1 kb DNA ladder for (A) and λ DNA/PstI DNA ladder for (B).

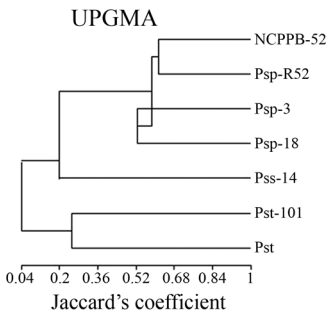


**Figure 5** - Approximate relationships among *P. syringae* pathovars based on UPGMA analysis of the combined data sets obtained with REP-PCR and BOX-PCR primers.

of *P. savastanoi* pv. *savastanoi* was particularly useful in the present study. Another set of primers (IAALF and IAALR for the *iaaL* gene) (Penyalver *et al.*, 2000) was also useful for identifying *P. savastanoi* pv. *savastanoi* strains from naturally infected and asymptomatic olive trees. The use of combinations of modified primers in REP-PCR enhanced the range of DNA fingerprints that could be detected. In this approach, it is the reverse primers rather than the forward primers that bind to the different annealing sites in the genome to produce the diversity of DNA amplicons.

The discriminatory potential of rep-PCR for different *P. syringae* pathovars has been shown in previous reports (Louws *et al.*, 1994; Weingart and Völksch, 1997; Stead *et al.*, 2004; Vicente and Roberts, 2007; Kaluzna *et al.*, 2010). A pathovar is defined as a subspecific group of strains that can infect particular plants within a certain genus or species. Intra-pathovar diversity is generally of two types, namely, (a) isolates from the same pathovar that may have identical REP, BOX and ERIC fingerprints and (b) isolates that do not share common rep-PCR banding patterns (Louws *et al.*, 1994). The first of these two groups was initially identified in an analysis of samples that included *P. syringae* pv. *morsprunorum* and *Xanthomonas campestris* pv. *phaseoli*; in this case, the number of monomorphic REP-PCR bands was greater than that of polymorphic bands in 12 isolates of *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *syringae* and *P. syringae* pv. *tomato* from distant locations in the United States (Louws *et al.*, 1994). As shown here, the insertion of G and C in Rep2-Dt primers at positions 1, 4 and 11 yielded additional different sized bands in REP-PCR of *P. syringae* pv. *tomato* DNA (Figure 3). Despite the limited number of strains examined here, the use of modified REP-PCR primers enhanced the detection of intra-pathovar diversity by expanding the number of annealing sites. There was a clear difference in the the ability of the three types of rep-PCR to detect genetic diversity in *P. syringae*. Scortichini *et al.* (2004) reported that REP primers were highly discriminatory in distinguishing among *P. savastanoi* isolates from Italy. The *P. syringae* genome contains high copy numbers of REP elements that are related to insertion sequence elements (ISI) (Tobes and Pareja, 2006). This feature of REP elements may explain the abundance and diversity of fingerprints seen upon amplification of the intervening regions.

In our work, *P. s*. pv. *savastanoi* isolates showed more polymorphism than tomato and bean isolates by using REP primer combinations. One of the reasons for that is the bacterial population which was isolated from knots could be contained both epiphytic and pathogenic strains of *P. savastanoi.* In addition, *P. savastanoi* pv. *savastanoi* strains may have diverged extensively during their co-evolution with olive trees (*Olea europaea*) (Scortichini *et al.*, 2004), particularly since the latter are widely distributed and well adapted in western Turkey.

The BOXA1R universal primer identified genetically distinct *P. syringae* pathovars and could therefore be potentially useful as a diagnostic tool, although the number of fingerprints it yielded was considerably lower than that obtained with REP-PCR. No inter-ERIC fingerprints were obtained for any of the isolates. Although they have been used in *P. syringae* genotyping (Louws *et al.*, 1994; Scortichini *et al.*, 2004; Vicente and Roberts, 2007; Kaluzna *et al.*, 2010), ERIC sequences have been found only in the Enterobacteriaceae and *Vibrio* species (Wilson and Sharp, 2006). Theoretically, ERIC primers bind to sequences between ERIC copies and produce fragments of variable length, depending on the positions of these conserved elements. The amplifications reported for ERIC-PCR in several reports may have arisen from randomly binding primers and consist of homologous bands shared by isolates or pathovars. The random nature of ERIC-PCR can also be seen in the successful amplification of fungal DNA (Gürel *et al.*, 2010), even though ERIC elements have not yet been reported in fungal DNA.

The availability of low-cost, fast and reliable methods for screening genetic diversity is a valuable tool in the diagnosis of local bacterial populations. The work described here provides a procedure for the isolation of *P. savastanoi pv. savastoni* isolates from olive knots and for reliable identification using species-specific primers. The use of modified primers allows the genetic variation among isolates in a particular environment to be monitored in a short time by colony PCR. The most important advantage in this approach is that the primer combinations enhance the possibility of obtaining distinct and unique banding patterns among the genotypes that can extend our understanding of the genetic diversity of these organisms. As shown here, the banding patterns obtained with a cytosine-containing primer (Rep2-C) yielded information on genetic relationships and led to the identification of distinct pathovar groups (Figure 5). Variations based on extragenic repetitive DNA in local isolates of *P. savastanoi* may provide clues to the nature of mutations associated with REP elements and the importance of the latter as regulators of bacterial gene expression.

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## References

Basim H and Ersoy A (2001) Identification of *Pseudomonas savastanoi* pv. *savastanoi*, olive knot pathogen, by polymerase chain reaction. In: Abstracts, Phytopathology Salt Lake 2001 APS/SON/MSA Joint Meeting, 25-29 August, 2001, Abstract S6.

Bereswill S, Bugert P, Völksch B, Ullrich M, Bender CL and Geider K (1994) Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. Appl Environ Microbiol 60:2924-2930.

Cirvilleri G, Scuderi G, Bonaccorsi A and Scortichini M (2006) Molecular characterization of *Pseudomonas syringae p*v. *syringae* strains from different host plants using fluorescent amplified fragment length polymorphism. J Plant Pathol 88:327-331.

de Bruijn FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180-2187.

Gardan L, Shafik H, Belouin S, Broch R, Grimont F and Grimont PAD (1999) DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). Int J Syst Bacteriol 49:469-478.

Gürel F, Albayrak G, Diken O, Çepni E and Tunali B (2010) Use of Rep-PCR for genetic diversity analyses in *Fusarium culmorum.* J Phytopathol 92:781-787.

Higgins CF, Ames GFL, Barnes WM, Clement JM and Hofnung M (1982) A novel intercistronic regulatory element of prokaryotic operons. Nature 298:760-762.

Hirano SS and Upper CD (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - A pathogen, ice nucleus, and epiphyte. Microbiol Mol Biol Rev 64:624-653.

Jaccard P (1908) Nouvelles recherches sur la distribution florale. Bull Soc Vandoise Sci Nat 44:223-270.

Judd AK, Schneider M, Sadowsky MJ and de Bruijn FJ (1993) Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl Environ Microbiol 59:1702-1708.

Kado CI and Heskett MG (1970) Selective media isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas.* Phytopathology 60:969-976.

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

King GJ (1989) Plasmid analysis and variation in *Pseudomonas syringae*. J Appl Bacteriol 67:489-496.

King EO, Ward NK and Raney DE (1954) Two simple media for the detection of pyocyanin and fluorescein. J Lab Clin Med 44:301-307.

Louws FJ, Fulbright DW, Stephens CT and de Bruijn FJ (1994) Specific genomic fingerprints of phytopathogenic

*Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl Environ Microbiol 60:2286-2295.

Louws FJ, Rademaker JLW and de Bruijn FJ (1999) The three Ds of PCR-based genomic analysis of phytobacteria: Diversity, detection, and disease diagnosis. Annu Rev Phytopathol 37:81-125.

Manceau C and Horvais A (1997) Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato.* Appl Environ Microbiol 63:498-505.

Mosqueda-Cano G and Herrera-Esterella L (1997) A simple and efficient PCR method for specific detection of *Pseudomonas syringae* pv. *phaseolicola* in bean seeds. World J Microbiol Biotechnol 13:463-467.

Murillo J and Sesma A (2001) The biochemistry and molecular genetics of host range definition: *Pseudomonas syringae.* J Phytopathol Medit 40:3-26.

Oguiza JA, Rico A, Rivas LA, Sutra L, Vivian A and Murillo J (2004) *Pseudomonas syringae* pv. *phaseolicola* can be separated into two genetic lineages distinguished by the possession of the phaseolotoxin biosynthetic cluster. Microbiology 150:473-482.

Penyalver R, Garcia A, Ferrer A, Bertolini E and Lopez MM (2000) Detection of *Pseudomonas savastanoi* pv. *savastanoi* in olive plants by enrichment and PCR. Appl Environ Microbiol 66:2673-2677.

Quesada JM, Perez-Martinez I, Ramos C, Lopez MM and Penyalver R (2008) IS53: An insertion element for molecular typing of *Pseudomonas savastanoi* pv. *savastanoi*. Res Microbiol 159:207-215.

Saygili H (1995) Fitobakteriyoloji. E.U. Ziraat Fakültesi Bitki Koruma Bölümü, Izmir, Turkey, 9944-5822-0-2.

Scortichini M, Rossi MP and Salerno M (2004) Relationship of genetic structure of *Pseudomonas savastanoi* pv. *savastanoi* populations from Italian olive trees and patterns of host genetic diversity. Plant Pathol 53:491-497.

Sisto A, Cipriani MG, Tegli S, Cerboneschi M, Stea G and Santilli E (2007) Genetic characterization by fluorescent AFLP of *Pseudomonas savastanoi* pv. *savastanoi* strains isolated from different host species. Plant Pathol 56:366-372.

Stead DE (1992) Grouping of plant pathogenic and some other *Pseudomonas* species by using cellular fatty acid profiles. Int J Syst Bacteriol 42:281-295.

Stead DE, Simpkins SA, Weller SA, Hennesy J, Aspin A, Stanford H, Smith NC and Elphinstone JG (2004) Classification and identification of plant pathogenic *Pseudomonas* species by REP-PCR derived genetic fingerprints. In: Iacobellis NS, Collmer A, Hutcheson SW, Mansfield JW, Morris CE, Murillo J, Schaad NW, Stead DE, Surico G and Ullrich MS (eds) *Pseudomonas syringae* and Related Pathogens. Biology and Genetics. Kluwer Academic Publishers, Dordrecht, pp 411-420.

Tobes R and Pareja E (2006) Bacterial repetitive extragenic palindromic sequences are DNA targets for insertion sequence elements. BMC Genomics 7:e62.

Van Zyl E and Steyn PL (1990) Differentiation of phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars by numerical taxonomy and protein gel electrophoregrams. Syst Appl Microbiol 13:60-71.

Versalovic J, Koeuth T and Lupski RJ (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823-6831.

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

Vicente J and Roberts S (2007) Discrimination of *Pseudomonas syringae* isolates from sweet and wild cherry using rep-PCR. Eur J Plant Pathol 117:383-392.

Weingart H and Völksch B (1997) Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. J Phytopathol 145:339-345.

Wilson LA and Sharp PM (2006) Enterobacterial repetitive intergenic concensus (ERIC) sequences in *Escherichia coli*: Evolution and implications for ERIC-PCR. Mol Biol Evol 23:1156-1168.

Woods CR, Versolovic J, Koeuth T and Lupski JR (1993) Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationship of bacterial isolates. J Clin Microbiol 31:1927-1931.

Young JM, Saddler GS, Takikawa Y, De Boer SH, Vauterin L, Gardan L, Gvozdyak R and Stead DE (1996) Names of plant pathogenic bacteria 1864-1995. Rev Plant Pathol 75:721-763.

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