Genetic Differentiation of *Pseudomonas syringae* Pathovar *tomato* from Other *P. syringae* Pathovars using REP-PCR and URP-PCR

Min Seok Cho^{1†}, Dong Suk Park^{1†}, Yeo Hong Yun², Seong Hwan Kim^{2*}, Myung Yong Shim³, Chang Won Choi⁴ and Young Shick Kim⁵

¹National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Korea

²Department of Microbiology and Institute of Basic Sciences, Dankook University, Cheonan, Chungnam 330-714, Korea

³Institute of Ecological Phytochemistry, Hankyong National University, Anseong, Kyonggi 456-749, Korea

⁴Department of Biology and Medicinal Science, Paichai University, Daejeon 302-735, Korea

⁵Department of Plant Science and Technology, Sangmyung University, Cheonan, Chungnam 330-720, Korea

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For the genetic differentiation of *Pseudomonas syringae* pathovar *tomato*, a total of 51 *P. syringae* pv. strains infecting 33 different host plants were analyzed using repetitive element PCR (REP-PCR) and universal rice primer PCR (URP-PCR). The entire DNA fingerprint profiles were analyzed using unweighted pair-group method with arithmetic averages (UPGMA). The 51 *P. syringae* pv. strains could be divided into five clusters based on 65% similarity by Rep-PCR using BOX, ERIC, and REP primers. *P. syringae* pv. *tomato* cluster was well separated from other 31 *P. syringae* pathovars. *P. syringae* pv. *tomato* cluster included only *P. syringae* pv. *maculicola* and *P. syringae* pv. *tomato*. *P. syringae* pv. *tomato* strains could be divided into two genetic groups. Meanwhile, the *Pseudomonas* pv. strains could be divided into four clusters based on 63% similarity by URP-PCR using 2F, 9F, and 17R primers. *P. syringae* pv. *tomato* cluster was also well separated from 30 other *P. syringae* pathovars. In this case, *P. syringae* pv. *tomato* cluster included *P. syringae* pv. *maculicola*, *P. syringae* pv. *berberidi*, and *P. syringae* pv. *tomato*. *P. syringae* pv. *tomato* strains was also separated into two genetic groups by URP-PCR analysis. Overall, our work revealed that *P. syringae* pv. *tomato* can be genetically differentiated from other *P. syringae* pathovars by the DNA fingerprint profiles of REP-PCR and URP-PCR. We first report that there are two genetically diverged groups in *P. syringae* pv. *tomato* strains.

Keywords : Genetic differentiation, *Pseudomonas syringae* pathovar *tomato*, REP-PCR, URP-PCR

Pseudomonas syringae is a group of Gram-negative bacteria that cause diseases on diverse plant species. It was first reported in 1894 as a type genus by Migula and has been classified in phylum *gracilicute*, class *proteobacteria*, order *pseudomonadales*, and family *pseudomonadaceae* (Murray, 2001). With the 1980 publication of the Approved Lists of Bacterial Names (Skerman et al., 1989), all plant pathogenic bacteria that resembled *P. syringae* were amalgamated into this species regardless of host range. Thus, to overcome instability of bacterial classification and to give relatively importance on pathogenicity, the term pathovar has been used to refer to *Pseudomonas* strains with similar features that are differentiated at the subspecies level on the basis of differences in plant host range and types of symptoms, and additionally by biochemical profiles (Dye et al., 1980; Schaad et al., 2001). So far, more than 50 pathovars have been reported in *P. syringae*. But discrimination of isolates at the pathovar level is not reliable for some pathovars of *P. syringae* because phenotypic, nutritional and genetic characteristics of strains tend to be inconsistent and conflicting and there is problem of relying on distinct host specificity to describe pathovars (Wiebe and Campbell, 1993). Thus, to overcome the difficulty in defining *P. syringae* pathovars it would be valuable to understand the genetic diversity and relationships of *P. syringae* pathovars.

Among *P. syringae* pathovars, *P. syringae* pv. *tomato* is particularly detrimental to tomato plants, where it causes a disease known as bacterial speck. This can either decrease crop yield by inducing foliar necrosis or blemish the fruits making them unsuitable for the fresh market or peeled tomato industry (Schneider and Grogan, 1977). So far, not only *P. syringae* pv. *tomato* but also several other *P. syringae* pathovars such as *P. syringae* pv. *maculicola*, *P. syringae* pv. *antirrhini*, *P. syringae* pv. *passiflorae*, and *P. syringae* pv. *apii* have been known to be pathogenic to tomato plants (Manceau and Horvais, 1997). Since these *P. syringae* pathovars are genetically closely related and strains of *P.*

[†]Equally contributed.

*Corresponding author.

Phone) +82-41-550-3454, FAX) +82-41-523-3454

E-mail) piceae@dankook.ac.kr

Table 1. *Pseudomonas syringae* pathovar strains used in this study

No.	Pathovar	Strain	Host	Geographic origin
1	<i>tomato</i>	LMG5093 ^(T)	<i>Solanum lycopersicum</i>	United Kingdom
2	<i>tomato</i>	OH314*	<i>Solanum lycopersicum</i>	United States
3	<i>tomato</i>	LMG5507	<i>Solanum lycopersicum</i>	Canada
4	<i>tomato</i>	LMG5155	<i>Solanum lycopersicum</i>	United States
5	<i>tomato</i>	LMG5395	<i>Solanum lycopersicum</i>	Denmark
6	<i>tomato</i>	LMG5506	<i>Solanum lycopersicum</i>	Yugoslavia
7	<i>tomato</i>	LMG5508	<i>Solanum lycopersicum</i>	Switzerland
8	<i>tomato</i>	LMG5509	<i>Solanum lycopersicum</i>	New Zealand
9	<i>tomato</i>	DC84-1*	<i>Solanum lycopersicum</i>	Canada
10	<i>tomato</i>	188B*	<i>Solanum lycopersicum</i>	Canada
11	<i>tomato</i>	DC89-4H*	<i>Solanum lycopersicum</i>	Canada
12	<i>tomato</i>	JL1035*	<i>Solanum lycopersicum</i>	United States
13	<i>tomato</i>	SM78-1*	<i>Solanum lycopersicum</i>	United States
14	<i>tomato</i>	PST 26L*	<i>Solanum lycopersicum</i>	South Africa
15	<i>tomato</i>	AV80*	<i>Solanum lycopersicum</i>	United States
16	<i>tomato</i>	3357*	<i>Solanum lycopersicum</i>	New Zealand
17	<i>tomato</i>	487*	<i>Solanum lycopersicum</i>	Greece
18	<i>maculicola</i>	LMG5071 ^(T)	<i>Brassica oleracea</i>	New Zealand
19	<i>maculicola</i>	LMG5150	<i>Brassica oleracea</i>	United Kingdom
20	<i>maculicola</i>	LMG2208	<i>Brassica oleracea</i>	United States
21	<i>antirrhini</i>	NCPPB1817 ^(T)	<i>Antirrhinum majus</i>	United Kingdom
22	<i>berberidis</i>	NCPPB2724 ^(T)	<i>Berberis</i> sp.	New Zealand
23	<i>persicae</i>	NCPPB2761 ^(T)	<i>Prunus persica</i>	France
24	<i>lachrymans</i>	NCPPB2916	<i>Cucumis melo</i>	Zimbabwe
25	<i>atrofaciens</i>	LMG5000	<i>Triticum aestivum</i>	Canada
26	<i>ciccaronei</i>	LMG5541 ^(T)	<i>Ceratonia siliqua</i>	Italy
27	<i>delphinii</i>	KACC10394 ^(T)	<i>Delphinium</i> sp.	New Zealand
28	<i>dysoxyli</i>	LMG5062 ^(T)	<i>Dysoxylum spectabile</i>	New Zealand
29	<i>eriobotryae</i>	LMG2184 ^(T)	<i>Eriobotrya japonica</i>	United States
30	<i>garcae</i>	LMG5064 ^(T)	<i>Coffea arabica</i>	Brazil
31	<i>glycinea</i>	KACC10393 ^(T)	<i>Glycine max</i>	New Zealand
32	<i>helianthi</i>	LMG2198	<i>Helianthus annuus</i>	Zambia
33	<i>japonica</i>	LMG5068 ^(T)	<i>Hordeum vulgare</i>	Japan
34	<i>lapsa</i>	LMG2206 ^(T)	<i>Zea</i> sp., hybrid seed	NK
35	<i>mellea</i>	LMG5072 ^(T)	<i>Nicotiana tabacum</i>	Japan
36	<i>mori</i>	LMG5074 ^(T)	<i>Morus alba</i>	Hungary
37	<i>morsprunorum</i>	LMG5075 ^(T)	<i>Prunus domestica</i>	NK
38	<i>myricae</i>	LMG5668 ^(T)	<i>Myrica rubra</i> , stem gall	Japan
39	<i>oryzae</i>	KACC10133 ^(T)	<i>Oryza sativa</i>	Japan
40	<i>panici</i>	LMG2367 ^(T)	NK	NK
41	<i>papulans</i>	LMG5076 ^(T)	<i>Malus pumila</i>	Canada
42	<i>passiflorae</i>	LMG2237	<i>Passiflora edulis</i>	New Zealand
43	<i>pisi</i>	LMG5383	<i>Pisum sativum</i>	Canada
44	<i>primulae</i>	LMG2252 ^(T)	<i>Primula</i> sp.	United States
45	<i>ribicola</i>	LMG2276 ^(T)	<i>Ribes aureum</i>	NK
46	<i>sesami</i>	KACC10649 ^(T)	NK	Yugoslavia
47	<i>syringae</i>	KACC10134 ^(T)	<i>Syringa vulgaris</i>	United Kingdom
48	<i>tabaci</i>	KACC10388 ^(T)	<i>Nicotiana tabacum</i>	Hungary
49	<i>tagetis</i>	KACC10389 ^(T)	<i>Tagetes erecta</i>	Zimbabwe
50	<i>ulmi</i>	LMG2249 ^(T)	<i>Ulmus</i> sp.	Yugoslavia
51	<i>viburni</i>	LMG2351 ^(T)	<i>Viburnum</i> sp.	United States

^(T) : Type-strain.

NK: not known.

BCCM/LMG : Belgian Co-ordinated Collections of Microorganisms, Belgium.

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NCPPB : National Collection of Plant Pathogenic Bacteria, York, United Kingdom.

KACC : Korean Agricultural Culture Collection, Suwon, Republic of Korea.

syringae pv. *tomato* also caused disease on cauliflower (Cuppels and Ainsworth, 1995; Razo, 1987), discrimination of *P. syringae* pv. *tomato* from other tomato-infecting *P. syringae* pathovars is not easy. In addition, some other *P. syringae* pathovars also have similar genotype to *P. syringae* pv. *tomato* (Cuppels and Ainsworth, 1995; Gardan et al., 1999), differentiation of *P. syringae* pathovars from infected tomato is confusing and challenging.

DNA fingerprinting has been extensively used for the delineation of species, subspecies, and pathovars (Louws et al., 1994). Especially, repetitive extragenic palindromic (REP)-, enterobacterial repetitive intergenic consensus sequence (ERIC)- and a subunit of the BOX element (Martin et al., 1992) sequence (BOX)-primed PCR have previously been used in studies on pathovar identification for strains of *Pseudomonas*, including *P. syringae* pv. *tomato*, *P. syringae* pv. *maculicola*, and other bacterial genera (Louws et al., 1994; Zhao et al., 2000; Scortichini et al., 2003). Recently, another type of rep-PCR, URP-PCR that are developed from repetitive sequences in the rice genome, has been used to fingerprint genomes of diverse organisms including plants, animals, fungi and bacteria (Kang et al., 2002; Jana et al., 2005). The URP-PCR uses long primers (20 nucleotides) and highly stringent PCR conditions in contrast to randomly amplified polymorphic DNA (RAPD) and arbitrary primer (AP)-PCR techniques. However, there has been no comparative assessment that whether these two rep-PCR-driven DNA fingerprints really well consistently resolve or not the genetic differentiation of *P. syringae* pv. *tomato* from other *P. syringae* pathovars. Therefore, this study was carried out to assess the genetic relatedness of *P. syringae* pv. *tomato* strains. Here, we report that rep-PCR-driven DNA fingerprints would vary depending on rep-primers used, and thus the DNA fingerprints allow us to genetically differentiate *P. syringae* pv. *tomato* from other *P. syringae* pathovars. In addition, we first report that there are two genetically diverged groups in

P. syringae pv. *tomato* strains.

A total of fifty one strains of various *Pseudomonas syringae* pathovars were analyzed in this study (Table 1). The strains were cultured and maintained according to the protocols described by Schaad et al. (2001). Bacterial genomic DNA was prepared as described by Pitcher et al. (1989). For the DNA isolation, the bacterial strains were cultured on LB broth for 24 to 48 hour at 28°C and harvested in 1.5 ml microtube through centrifugation at room temperature. The harvested bacterial cells were suspended with 500 µl of suspension buffer (0.15 M NaCl, 0.01 M EDTA, pH 8.0) and the suspension was centrifuged for 3 min at 13,000 g. The precipitated bacterial cells were suspended with 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), mixed with 500 µl guanidine thiocyanate - EDTA - Sarkosyl (GES) solution (per 100 ml: 60 g guanidine thiocyanate, 20 ml 0.5 M EDTA pH 8.0, 20 ml sterile water, 1 g N-lauroyl sarkosine) and then, treated for 5 min on ice chips. It was mixed well with 250 µl of 7.5 M ammonium acetate, stored at -20°C, then reacted for 5 min on ice chips. Chloroform/iso-amyl-alcohol (24:1, v/v) was added, mixed well, and then centrifuged for 10 min. at 13,000 g. The supernatant was transferred to a new microfuge tube and precipitated with isopropanol. The precipitate was washed with 70% ethanol, resolved in TE buffer and treated with 25 µl RNase (250 µg/ml). After cleaned with a DNA clean-up kit (Qiagen), the purified genomic DNA was quantified with Nanodrop ND-1000 Spectrophotometer (Ver 4.3, Wilmington, USA) and stored at -20°C until use.

Genomic fingerprinting was carried out according to the methods of Rep-PCR (with BOX, ERIC, and REP primers) of Louws et al. (1994) and URP-PCR (URP 2F, 9F, and 19R primers) of Kang et al. (2002). PCR reaction was carried out with PTC-200™ DNA Engine thermocycler (MJ Research Inc, Watertown, MA, USA) in 50 µl volumes containing 50 ng of genomic DNA, 2.5 unit *Taq* DNA

Table 2. Oligo-primers used for DNA fingerprinting in this study

Primer	Primer sequence (5'-3')	References
For Rep-PCR		Louws et al., 1994; Weingart and Volksch, 1997
BOX A1R	CTACGGCAAGGCGACGCTGACG	
ERIC 1R	ATGTAAGCTCCTGGGGATTAC	
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	
REP 1R	IIICGICGICATCIGGC	
REP 2I	ICGICTTATCIGGCCTAC	
For URP-PCR		Kang et al., 2002
URP 2F	GTGTGCGATCAGTTGCTGGG	
URP 9F	ATGTGTGCGATCAGTTGCTG	
URP 17R	AATGTGGGCAAGCTGGTGGT	

Table 3. PCR conditions set for the primers used in this study

BOX	REP	ERIC	URP	cycle
95°C for 7 min	95°C for 7 min	95°C for 7 min	94°C for 4 min	1
94°C for 1 min	94°C for 1 min	94°C for 1 min	94°C for 1 min	–
53°C for 1 min	40°C for 1 min	52°C for 1 min	55°C for 1 min	35
65°C for 8 min	65°C for 8 min	65°C for 8 min	72°C for 2 min	–
65°C for 16 min	65°C for 16 min	65°C for 16 min	72°C for 7 min	1
4°C for 20 min	4°C for 20 min	4°C for 20 min	4°C for 20 min	1

Polymerase (Promega, Madison, Wisconsin, USA), and final concentration of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 µM dNTP, and 200ng of respective primers. Primers used in this study were BOX A1R, ERIC 1R, ERIC 2, REP 1R, and REP 2I for rep-PCR and URP-2F, URP-9F, and URP-19R for URP-PCR (Table 2). The cycle conditions were given in Table 3. The amplified PCR products were analyzed by gel electrophoresis on a gel containing 1.5% agarose in 1 × TAE buffer. The gels were stained with ethidium bromide and destained with DW, then viewed and photographed with VersaDoc 1000 (Molecular Imager VersaDoc MP Imaging Systems, USA). Similarity analyses were done with the NTSYSpc ver. 2.02 software (Exeter Software, New York, USA) (Rohlf, 1998). Similarity coefficients were compared using DICE coefficient analysis according to number and position of bands (Dice, 1945). Dendrograms were produced according to the unweighted pair-group mean arithmetic method (UPGMA) using NTSYSpc software.

DNA fingerprints were produced against all bacterial strains used using BOX, ERIC, REP, URP 2F, URP 9F, and URP 17R primers (Figs. 1 and 2). BOX primers generated 200 to 7000 bp size bands and ERIC or REP primers produced 100 to 6000 bp size bands. Meanwhile, 100 to 4000 bp size bands were amplified with URP 2F primer and 100 to 30000 bp size bands with URP 9F or 17R primer. These results showed that the size of amplified PCR products varied depending on the primers used. Since the resolution of band patterns would be more clear with diverse ranges of band size, we thought that the combinational use of different primers which could generate different sizes of bands could lead to analyze genetic differentiation of *Pseudomonas syringae* pathovar *tomato* strains from other closely related *P. syringae* pv. strains. Thus, we first analyzed DNA bands produced by BOX, ERIC, and REP primers. Among 17 *P. syringae* pv. *tomato* strains, 15 strains (named as GI group in Fig. 1) were separated well from other *P. syringae* pv. strains (Fig. 1). The remained three *P. syringae* pv. *tomato* strains, LMG5093, OH314, and LMG5507 (named as GII group in Fig. 1) clustered with *P. syringae* pv. *maculicola* and this cluster

was also well separated from other *P. syringae* pv. strains. Because it has been reported that *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* are closely related (Cuppels and Ainsworth, 1995; Henderson et al. 1992; Peters et al., 2004) and suggested as synonyms of one pathovar by some authors (Palleroni, 1984; Takikawa et al., 1994), the clustering of *P. syringae* pv. *tomato* strains LMG5093, OH314, and LMG5507 with three *P. syringae* pv. *maculicola* strains and their positioning in the neighbor of *P. syringae* pv. *tomato* (GI group) support earlier reports on their relatedness. Within this cluster, three *P. syringae* pv. *tomato* strains LMG5093, OH314, and LMG5507 could be separated from *P. syringae* pv. *maculicola* strains. This separation suggests that although *P. syringae* pv. *tomato* GII group and *P. syringae* pv. *maculicola* have close genetic relationships, they could be genetically divided. In general, *P. syringae* pv. *tomato* do not cause disease on Brassicaceae plant such as broccoli and cauliflower. On the contrary, *P. syringae* pv. *maculicola* causes disease not only on Brassicaceae but also on tomato (Cuppels and Ainsworth, 1995). Thus, genetic separation of *P. syringae* pv. *maculicola* from both the GI or GII group of *P. syringae* pv. *tomato* strains using REP-PCR is meaningful for the diagnosis of bacterial disease caused by these pathovars.

When we analyzed DNA fingerprint patterns generated with URP-2F, -9F, and -17 primers, fifteen *P. syringae* pv. *tomato* strains (named as GI group in Fig. 2) were also separated well from other *P. syringae* pathovar strains (Fig. 2). Although there are some variations in the tree topology and in genetic relatedness among the fifteen *P. syringae* pv. *tomato* strains, the strain names of pathovars belonging to the GI group in Fig. 1 and those of the GI group in Fig. 2 were the same. It is noticeable that although the fifteen *P. syringae* pv. *tomato* strains were from different geographic origins, they formed one cluster both in Fig. 1 and 2. Our results indicate that both DNA fingerprint methods using the BOX, ERIC, and REP-primer based PCR and URP-primer based PCR are very useful tools for the genetic differentiation of *P. syringae* pv. *tomato* strains (that infect tomato as only host) from other *P. syringae* pathovars. *P. syringae* pv. *tomato* strains LMG5093, OH314, and LMG5507

Fig. 1. UPGMA dendrogram constructed from the combined Rep-PCR DNA fingerprints of the *P. syringae* pathovar groups. The normalized banding patterns representing BOX, ERIC, REP primers are found adjacent to each branch. Number in the inner branch of the dendrogram indicates genetic similarity.

Fig. 2. UPGMA dendrogram constructed from the combined URP-PCR DNA fingerprints of the *P. syringae* pathovar groups. The normalized banding patterns representing 2F, 9F, 17R primers are found adjacent to each branch. Number in the inner branch of the dendrogram indicates genetic similarity.

positioned as its own cluster in the genetic relatedness tree and, thus, we named them as GII group as like in Fig. 1 (Fig. 2). Meanwhile, all three *P. syringae* pv. *maculicola* strains did cluster not with *P. syringae* pv. *tomato* GII group strains but with *P. syringae* pv. *berberidis*, and positioned in the neighbor of *P. syringae* pv. *tomato* GI group strains. This result showed that the genetic relationships of *P. syringae* pv. *maculicola* to *P. syringae* pv. *tomato* GII group strains shown in Fig. 2 differed from that was shown in Fig. 1. From the results of Figs. 1 and 2, we propose that there are two distinct genetic groups within *P. syringae* pv. *tomato* strains. We named these two groups as GI and GII, respectively. This is first report of the differentiation of two divergent genetic groups in *P. syringae* pv. *tomato* strains.

Recently, Gardan et al. (1999) performed DNA relatedness analysis using DNA-DNA hybridization and ribotyping methods and grouped 48 *P. syringae* pathovars into nine genomospecies. In their report, *P. syringae* pv. *tomato* was grouped in genomospecies 3 with *P. syringae* pv. *persicae*, *P. syringae* pv. *antirrhini*, *P. syringae* pv. *maculicola*, *P. syringae* pv. *viburni*, *P. syringae* pv. *berberidis*, *P. syringae* pv. *apii*, *P. syringae* pv. *delphinii*, *P. syringae* pv. *passiflorae*, *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *lachrymans*, *P. syringae* pv. *philadelphia*, *P. syringae* pv. *ribicola*, and *P. syringae* pv. *primulae*. When we compared their results with our Fig. 1 results from the genetic relatedness analysis by REP-PCR, among the genomospecies 3, only *P. syringae* pv. *maculicola*, *P. syringae* pv. *persicae*, *P. syringae* pv. *antirrhini*, and *P. syringae* pv. *berberidis* were closely related to *P. syringae* pv. *tomato*. However, *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *passiflorae*, *P. syringae* pv. *primulae*, and *P. syringae* pv. *ribicola* of the genomospecies 3 were very distantly related with *P. syringae* pv. *tomato*. On the other hand, when we compared the work of Gardan et al. (1999) with our Fig. 2 results from the genetic relatedness analysis by URP-PCR, among the genomospecies 3, *P. syringae* pv. *persicae*, *P. syringae* pv. *antirrhini*, *P. syringae* pv. *maculicola*, *P. syringae* pv. *viburni*, *P. syringae* pv. *berberidis*, *P. syringae* pv. *morsprunorum*, and *P. syringae* pv. *passiflorae* were closely related pathovars to *P. syringae* pv. *tomato*. But *P. syringae* pv. *delphinii*, *P. syringae* pv. *lachrymans*, *P. syringae* pv. *primulae* and *P. syringae* pv. *ribicola* of the genomospecies 3 were distantly related with *P. syringae* pv. *tomato*. Thus, we found that URP-PCR results better agreed with the work of Gardan et al. (1999) than REP-PCR results.

Manceau and Horvais (1977) assessed the genetic diversity of 30 *P. syringae* pathovars by PCR-RFLP analysis and divided the pathovars into 18 RFLP groups (from A to Q group). In the 18 RFLP-groups, they put *P. syringae* pv. *tomato* in the group A together with *P. syringae* pv. *antirrhini*, *P. syringae* pv. *berberidis*, *P. syringae* pv. *lachrymans*,

P. syringae pv. *maculicola*, *P. syringae* pv. *passiflorae*, and *P. syringae* pv. *persicae*. We could see that the close genetic relatedness among the *P. syringae* pathovars of this group A is well resolved not by REP-PCR analysis (Fig. 1) but by URP-PCR analysis (Fig. 2). These results also showed that URP-PCR results better agreed with the work of Manceau and Horvais (1977) than REP-PCR results.

In conclusion, we could differentiate *P. syringae* pv. *tomato* strains into two divergent genetic groups and separate them from other 36 *P. syringae* pathovars using BOX, ERIC, REP primers used in REP-PCR and URP 2F, URP 9F, and URP 17R primers used in URP-PCR. Overall, the DNA fingerprints analysis using REP-PCR and URP-PCR made us the comparison of the genetic relatedness of *P. syringae* pv. *tomato* to other *P. syringae* pathovars. When it comes to the consistency and support of the previous reports on genetic relatedness *P. syringae* pathovars, URP-PCR produced better DNA fingerprints than REP-PCR.

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