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Identification and genotyping fingerprinting of 2,4-DAPG producing *Pseudomonas* spp.

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PCR based DNA finger printings were performed by ISSR primers, gene specific primers (Plt) and primers derived from 16S ribosomal RNA. Fifteen primers pairs were used to detect the polymorphism between 45 isolates of *Pseudomonas* spp. Results revealed that clear polymorphism and similarity coefficient ranged from 0.00 to 0.44 with ISSR primers, 0.01 to 0.83 with gene specific primers and 0.22 to 1.00 in with 16S ribosomal RNA specific primers. No correspondence was observed between the ISSR based clustering and pyocinin and fluorescine expression. Individual cluster grouped different types of isolates irrespective of their expression for pyocyanin, fluorescein or siderophore production. Dendrogram generated by Plt (gene specific primer) based DNA finger printing revealed that four set of gene specific primer were screened on 45 isolates to identify the primer that produce the polymorphic fragment. The isolates shows clearly scorable polymorphism and similarity coefficient ranged from 0.01 to 0.83. DNA amplicons from all isolates differences in the molecular weight of the amplified product only formed similarity groups in subsequent sub-sub- clustering. Dendrogram generated by primers from 16S ribosomal RNA revealed that two set of 16S ribosomal RNA specific primer were screened on 45 isolates to identify the primer that produce the polymorphic fragment. The isolates show clearly scorable polymorphism and similarity coefficient ranging from 0.22 to 1.00. The isolates (P6, P76, P113, P82, P195, P136, P196, P20, P224, P133, P223, P14, P155 and P98); (P9, P16, P85, P70, P109, P217 and P144); (P16, P57, P98, P59, P138, P85, P179 and P184) formed different clusters but had no correspondence with the pigment production or biochemical tests.

Key words: *Pseudomonas* spp., genotyping fingerprinting.

INTRODUCTION

Pseudomonas fluorescens is a physiologically diverse species of opportunistic bacteria (gamma-proteobacteria) found throughout terrestrial habitats and ecologically significant group of culturable bacteria (Spiers et al., 2000). The species contributes greatly to the turnover of organic matter and, while present in soil, is abundant on the surfaces of plant roots and leaves. The mechanistic bases of these effects remain unclear, but are known to include the production of plant growth hormones, the suppression of pathogens (especially fungi and Oomycetes) detrimental to plant health via competitive and/or allelepathic effects, and the direct elicitation of plant defense responses. It has been argued that exploitation of these plant growth promoting bacteria in agriculture requires an improved understanding of the determinants of ecological performance, particularly persistence (Rainey, 1999). However, the ability to comprehensively identify ecologically important sequences was limited in these previous studies due to the presence of incomplete genome libraries and the lack of whole genome sequences. The

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Table 1. List of isolates of *Pseudomonas* spp. and code.

S. No.	Code	Name of place	S. No.	Code	Name of place
1	P6	IGKV Horticulture Cashew	24	P120	Jaisekara (tree root)
2	P9	IGKV Horticulture Turmeric	25	P123	Jungle 2
3	P14	Chatti T	26	P124	Jungle 2
4	P16	Dhamtari road T	27	P127	Jungle 3
5	P18	Jungle 3 T	28	P133	Jungle 3
6	P19	Kurud T	29	P136	Hirri Jugni (Jagdalpur)
7	P20	Purur T	30	P138	Kirda
8	P21	Satpara T	31	P144	Kodebor (paddy field)
9	P28	VIP Road T	32	P153	Kurud (rice gram field)
10	P29	VIP Road T	33	P155	Kurud (rice straw)
11	P44	Abhanpur(road)	34	P167	Purur
12	P52	Bhatagaon	35	P178	Hirri Jugni (Jagdalpur)
13	P57	Bhatagaon (degraded paddy straw)	36	P179	Rice lathyrus
14	P59	Bhatagaon (degraded paddy straw)	37	P184	Satpara Arhar rice field
15	P62	Charama	38	P195	VIP road
16	P70	Charama (paddy field)	39	P196	VIP road
17	P76	Chhati (mustard)	40	P201	VIP road
18	P82	Darba	41	P217	Darba
19	P85	Darba (cow dung)	42	P221	Badra chalam (Jagdalpur)
20	P98	Jagdalpur Kailash cave	43	P223	Jugani (Jagdalpur)
21	P102	Jagtara	44	P224	Konta (Jagdalpur)
22	P109	Jagtara forest	45	P226	Pareshgaon (Jagdalpur)
23	P113	Jaisekara			

genome sequence of a single isolate of *P. fluorescens*, Pf- 5, has been reported. Although, a large number of genes involved in nutrient uptake/degradation and biocontrol were identified in Pf-5, the true diversity within this species was not revealed. In addition to its use in the study of microbe-plant soil interactions, *P. fluorescens* has become an important model organism for studies on evolutionary processes.

Genes required for the synthesis of 2, 4-DAPG by *P. fluorescens* includes *phlA*, *phlC*, *phlB* and *phlD*; which are transcribed as an operon from a promoter upstream of *phlA* (Bangera and Thomashow, 1999). *PhlD* is responsible for the production of monoacetylphloroglucinol (MAPG), and *PhlA*, *PhlC*, and *PhlB* are necessary to convert MAPG to 2, 4-DAPG. The genetic diversity found within pseudomonads gives rise to a wide range of phenotypes. Increasing evidence suggests that the diversity of genome architecture (chromosomes and accessory genetic elements) is of particular importance (Jablonka et al., 1998; Kirschner and Gerhart, 1998; Gogarten et al., 2002). Examination of the deoxyribonucleic acid (DNA) fingerprints of *Pseudomonas* spp. reveals a high level of polymorphism among strains of a species and among strains highly related on a phenotypic basis (Ginard et al., 1997; Rainey et al., 1994). The ultimate cause of diversification is mutation, but the variation generated by both mutation and recombination (including lateral gene transfer) is sorted and shaped by selective pressure and genetic drift (Spiers et al., 2000). Due to the ubiquity and versatility of pseudomonads, there is considerable interest in exploiting these bacteria for a diverse range of biotechnological applications such as plant growth promotion and plant pest/disease control (Shah et al., 1998; Walsh et al., 2000; Shishido and Chanway, 2000; Tripathy et al., 2006).

Despite the extensive examination of *Pseudomonas*, there is a lack of knowledge concerning the degree and source of *Pseudomonas* spp. diversity.

MATERIALS AND METHODS

Isolation of florescent *Pseudomonas* from soil

45 *Pseudomonas* isolates (Table 1) from different geographical locations of Chhattisgarh namely, Chhati, Charama, Bhatagaon, Darba, Jagtara forest, Jungle (Kanker area), Kirda, Mana road (Raipur), Rajiv-gandhi marg (rice), Satpara paddy field, Dhamtari road (Raipur), VIP road (Raipur), IGKV Horticulture field and Abhanpur were collected and brought to the laboratory. Petri dishes were poured with 20 ml of King's B medium. For isolation and enumeration of fluorescent *Pseudomonas*, 100 µl of soil suspension from the final dilution (10^{-3}) was transferred in each plate and gently spread with glass L- shaped spreader to ensure uniform distribution of the soil sample. Plates were incubated at 28°C for 24 h. They were examined under UV light and colonies with yellow green and blue white color pigmentation were marked and recorded. Individual florescent colony was picked up with the help of sterilized loop and inoculated on solidified Kings medium B Base (KMB) (King et al.,

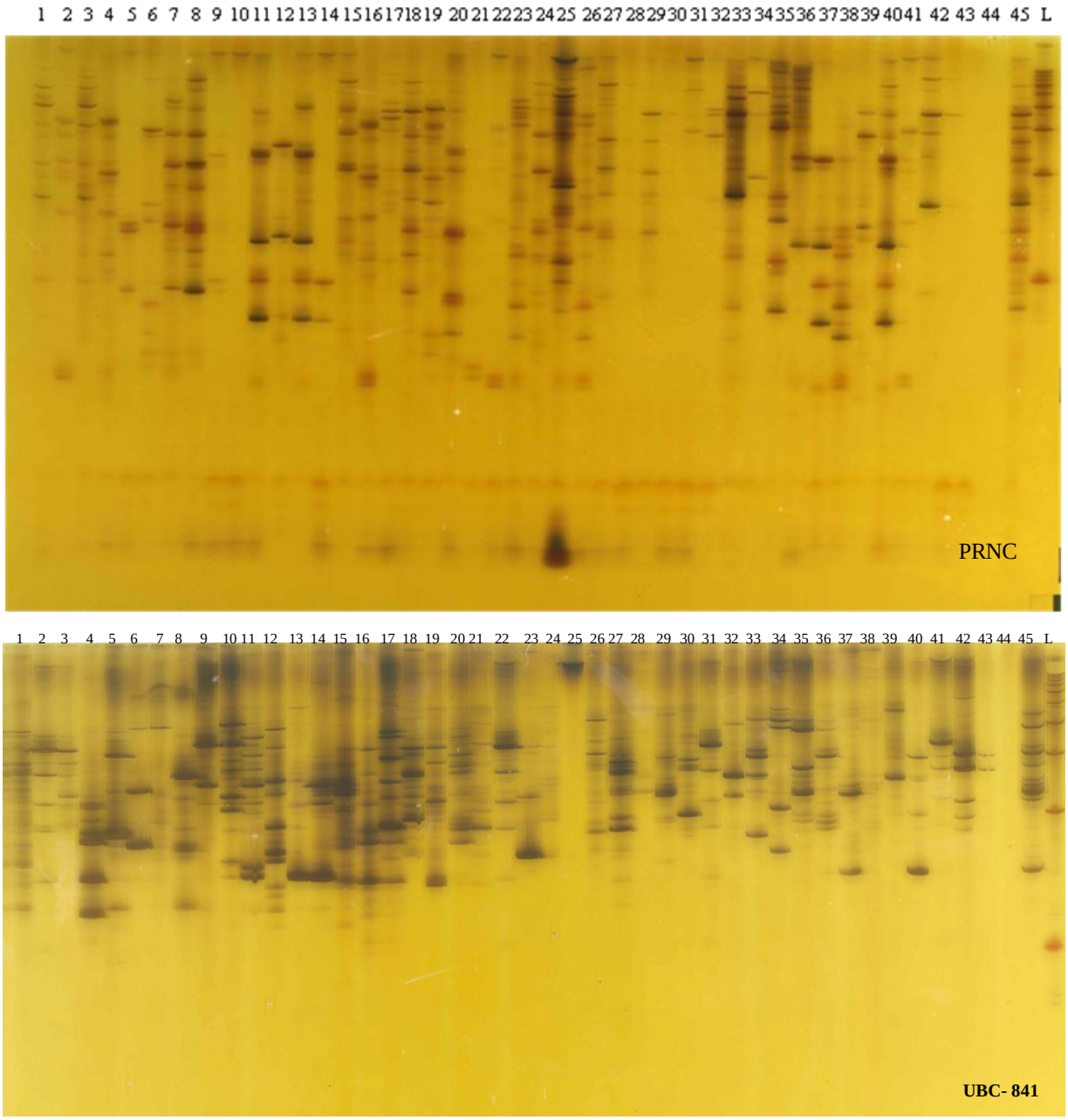

Plate 1. DNA finger printing of *Pseudomonas* isolates generated by PRNC and UBC primers using polyacrylamide gel electrophoresis (PAGE).

1954) by zigzag streaking. The plates were incubated at 28°C for 24 h. The colony growing at last tip of the zigzag line (which was well isolated and assumed to be developed from a few bacterial cells) was transferred to KMB slants. The isolates thus purified were given an identity as per the geographical location from where the soil sample was collected.

PCR based DNA fingerprinting of the isolates of fluorescent *Pseudomonas*

DNA finger printing of 45 *Pseudomonas* isolates from different geographical locations of Chhattisgarh were taken by polyacrylamide gel electrophoresis using ISSR and gene specific primers (*Plt*) and primers derived from 16s rRNA (Plate 1). Cluster analysis helped us to determine the genetic relationship among isolates.

DNA isolation of *Pseudomonas*

25 ml of King's medium 'B' Base (KMB) was inoculated with young viable cultures of *Pseudomonas.* Overnight grown cultures were centrifuged in 50 ml tarson tubes at 3000 rpm for 5 min (or in eppendorf tubes at 13000 rpm for 2 min). The pellet formed was resuspended in 4 ml 1% NaCl by pipetting. The suspensions were then transferred in 5 eppendorf tubes (750 µl each). They were then centrifuged at 12000 rpm for 1 min to form pellet. The pellet was resuspend in 750 µl of TES (10 mM Tris HCL 10 mM EDTA at pH

Table 2. Primer sequences of the markers used for DNA finger printing of 45 isolates of *Pseudomonas* spp.

Primer	Sequence	Target	Temperature (°C)
ISSR primers			
UBC 873	5'-GACAGACAGACAGACA-3'		45
UBC834	5'-AGAGAGAGAGAGAGAGYT-3'		45
UBC854	5'-TCTCTCTCTCTCTCTCRG-3'		52
UBC-856	5'-ACACACACACACACACYA-3'	Inter simple sequence repeat	45
UBC841	5'-GAGAGAGAGAGAGAGAYC-3'		45
ISSR-1	5'-CACACACACACAGG-3'		50
ISSR-2	5'-CTCTCTCTCTCTCTCTAC-3'		45
Intervening sequence-specific primers			
PltBf	5'-CGGAGCATGGACCCCCAGC-3'	*pltB*	65.3
PltBr	5'-GTGCCCGATATTGGTCTTGACCGAG-3'	*pltB*	66.3
Plt1	5'-ACTAAACACCCAGTCGAAGG-3'	*pltB*	50.2
Plt2	5'-AGGTAATCCATGCCCAGC-3'	*pltB*	57.9
PrnCf	5'-CCACAAGCCCGGCCAGGAGC-3'	prnC	67.6
PrnCr	5' GAGAAGAGCGGGTCGATGAAGCC-3'	prnC	66.0
Phl2a	5'-GAGGACGTCGAAGACCACCA-3'	Phl	61.4
Phl2b	5'-ACCGCAGCATCGTGTATGAG-3'	Phl	59.4
Primers derived from 16S ribosomal RNA gene			
EF15917	F-5'-TCCCTATCGATTGATCCGGCTTCT-3'		59.2
	R-5'-TTTAGATGGTGGAGCCAAGGAGGA-3'		59.4
AF36993	F-5'-ACGCTTTCTTTAAAGGGTGGCTGC-3'		60
	R-5'-TCTATCCATGGGCAGGTTGAAGGT-3'		59.7

8.0, 2% SDS) by pippeting and incubated at 75°C for 5 min to improve cell lysis. This was followed by adding 750 µl of water saturated phenol and was mixed by inverting the tubes for 5 min. Subsequently, the tubes were centrifuged at 12000 rpm for 3 min. The upper layer was removed and equal amount of chloroform: isomyl alcohol (24:1) was added and mixed by inverting the tubes for 5 min. This was followed by centrifugation at 12000 rpm for 7 min. Then the upper layer was transfer to new tubes and 1/10 vol of 5M potassium acetate (acetic acid buffer) and equal volume of iso-propanol alcohol was added (keep them in dip freezer for 15 min). The tubes were then inverted gently for 20 min and centrifuged at 12000 rpm for 10 min to form the pellets. The pellets were washed with 70% ethanol, recentrifuged at 12000 rpm for 3 min and then resuspended in 50 µl TE. The content was pooled in single eppendorf tube and 10 µl of RNase was added (10 mg/ml) and incubated at 37°C for 1 h.

The DNA was precipitated with twice the (400 µl) volume of absolute ethanol, incubated at -20°C for 30 min and centrifuged at 13000 rpm for 10 min. The pellets were then washed (centrifuge for 3 min) with 300 µl of 70% ethanol and air dried. At last, the pellets were dissolved in 200 µl of TE and stored at 4°C till use.

Quantification of DNA

The DNA sample was quantified using Nanodrop Spectrophotometer (ND 1000). 1 µl of isolated DNA was placed over tip of Nanodrop to record absorbance at 260 nm with TE as blank. The absorbance ratio (A260/A280) was recorded for each sample to estimate and to find out the purity of DNA. The acceptable absorbance ratio (A260/A280) for pure DNA is 1.8. After quantification, the DNA was diluted with TE such that final concentration was approximately 40 ηg/µl for analysis.

PCR analysis of 45 isolates of fluorescent *Pseudomonas*

PCR was performed using ISSR and gene based primers (Table 2). The following were the essential requirements for performing Polymerase Chain Reaction (PCR) in a thermal cycler. The reaction mixture and the temperature profiles used are summarized in Tables 3 and 4, respectively. 6% denaturing PAGE was performed to separate the PCR amplified products.

Visualizing AFLP products in denaturing polyacrylamide gel electrophoresis

Urea was first dissolved in 400 ml warm distilled water. Acrylamide bisacrylamide were weighed in fumehood and dissolved in 100 ml distilled separately and then added to the beaker containing urea solution. 100 ml of 10X TBE was added to the solution and the volume was made up to 1000 ml by adding autoclaved millipure water. The solution was sterilized by passing through 0.22-micron and stored in amber colour bottle at 4°C. Fix/stop solution was prepared for 1000 ml by mixing 100% ethanol 100 ml, glacial acetic acid 5 ml and Nanopure water 895 ml. Staining solutions was prepared for 1000 ml by mixing 2 g silver nitrate (final concentration

Table 3. Reaction mixture used for performing polymerase chain reaction (PCR) with different primers.

Component	Stock concentration	Volume/reaction
DNA	40 ng/µl	3 µl
ANW	-	10.5 µl
PCR buffer containing 15 Mm $MgCl_2$	10X	2 µl
dNTP	1 mM	2 µl
Forward primer	10 µM	1 µl
Reverse primer	10 µM	1 µl
Tag polymerase	1 U/µl	0.5 µl

Table 4. Temperature profile used for performing polymerase chain reaction (PCR) for different primers.

Step	Activity	Temperature (°C)		Duration (m)	Cycle
1	Initial denaturation	93		3	1
2	Denaturation	93		1	
		ISSSR-1	50		
		ISSSR-2 UBC856 UBC841 UBC-812	45		
3	Annealing	ISSR-52 UBC-854 UBC818	52	1	35
		AF 369903 EF 159157	61		
		Phl 2a/2b	60		
4	Extension	72		1	
5	Final extension	72		7	7
6	Storage	4		-	1

0.2%) and 1000 ml nanopure water. Developer was prepared by mixing 30 g sodium hydroxide (final concentration 3%), 5 ml formaldehyde (final concentration 0.5%) and final volume made up to 1000 ml with water. l0% solution of ammonium persuiphate (APS) was prepared by mixing 1.0 g of ammonium persuiphate and 10 ml of distilled water. Bind silane solution [3- (trimethoxysilyl) propylmethacrylate methacrylsaure-3 trimethoxysilyl propylester] of 1 ml was prepared by mixing 100% 945 µl ethanol, 5 µl bind sline, 5 µl glacial acetic acid and 45 µl ditilled water.

10 ml formamide dye was prepared by mixing 9.8 ml formamide (final concentration 98%), 200 µl of 0.5 M EDTA (final concentration 10 mM), 0.01 g xylene cyanol (final concentration 0.1%) and 0.01 g bromophenol blue (final concentration 0.1%).

PAGE procedure

The Bangalore genei sequencing gel apparatus was used for PAGE of ISSR and gene specific PCR products. The plates and spacers were cleaned thoroughly with three washes of distilled water followed by two washes with 75% alcohol and then with 100% alcohol, respectively. 1 ml bind silane solution was applied on unnotched plated, uniformly spread by wiping with a tissue paper, and kept for drying for 30 min. After drying, the plate was wiped with 100% alcohol. 1 ml of sigma cote (sigma, Cat. # SL-2) was applied to the notched plate, uniformly spread with a tissue paper and kept for drying for 30 min. Post-drying, the gel plates were assembled as per manufacturer's instructions so that the processed surfaces of both plates face each other. Then, the comb was inserted. 100 ml of the 6% urea-acrylamide gel solution, 450 µl of freshly prepared 10% ammonium persulphate (APS) solution and 45 µl TEMED were added and swirled gently. The solution was gently poured in the gap between the two glass plates by taking care to prevent the formation of air bubbles with the help of syringe and the gel was allowed to polymerize for at least an hour. After 1 h, the comb was removed gently without disrupting the gel line and the well was washed with distilled water by using a syringe. The gel

plates were fixed to the electrophoretic mold/gel tank and 1X TBE (running buffer) was added to the buffer chamber of the mold. The gels were put for pre run at 1200 V for 1 h, until the temperature of the plates reached 50°C and the gel line was washed with 1X TBE buffer. The comb was inserted again to form the wells by ensuring that the teeth of comb just touched the gel line uniformly.

The PCR reaction mixture were mixed with 3 µl of formamide dye and denatured at 95°C in PCR thermal cycler and immediately kept in ice. 3 µl of each denatured sample was loaded and the samples were electrophoresed at a constant 1200 V for 3.5 h. After completion of the run, the plates were allowed to cool to room temperature and then the bands were visualized by silver staining.

Silver staining

After electrophoresis, the power supply was disconnected, the comb was removed and the notched plate was separated from the unnotched plate. The gel adheres to the unnotched plate coated with bind silane. The plate transferred to a tray containing 2 L fix/stop solution and kept for 10 min at room temperature in the solution with shaking at 90 rpm. The gel was then given a quick wash with distilled water for 30 s. Then, the gel plate was transferred to a tray containing 0.2% silver nitrate solution, and kept for 10 min in dark with slow shaking, followed by a quick wash with distilled water for 30 s. The gel plate was then placed in the tray containing 2 L developer solution and kept for 5 to 7 min until bands began to appear. After bands with detectable intensity appeared, the gel was transferred to a tray containing distilled water and rinsed for few minutes. The gel was kept for air-drying and subsequently the bands were captured by scanning with HP scanner.

Data analysis

Specific amplification products were scored as present (1) or absent (0) depending on decreasing order of their molecular weights of DNA sample. The similarity matrix was calculated by UPGMA method and dendrogram was generated using SAHN subroutine of NTSYS (Rohlf, 1998).

RESULTS AND DISCUSSION

PCR based DNA finger printing of 45 selected isolates fluorescent *Pseudomonas*

PCR based finger printing is widely applied for the characterization of *Pseudomonas* strains and species for various purposes. Total genomic DNA was extracted from 45 selected isolates of fluorescent *Pseudomonas* (Plate 1). The PCR based DNA finger printings were performed by ISSR primers, gene specific primers (Plt) and primers derived from 16S ribosomal RNA. Data analysis was done by calculating similarity matrices using Numerical Taxonomy System Biostatistics (NTSYS) computer program. Total of 15 primers were used to detect the polymorphism between 45 isolates of *Pseudomonas* spp. and amplified bands per primer varied from 5 to 40. Isolates showed clear polymorphism and similarity coefficient ranged from 0.00 to 0.44 in dendrogram generated by the ISSR primers (Figure 1), 0.01 to 0.83 in the dendrogram generated by the gene specific primers (Figure 2) and 0.22 to 1.00 in case of set of 16S ribosomal RNA specific primers (Figure 3). RAPD and ISSR markers have been extensively used for DNA finger-printing (Moreno et al., 1998), genetic diversity studies (S'anchez et al., 1996; Esselman et al., 1999; Babalola et al., 2002; Ugur et al., 2012), population genetic studies (Wolfe et al., 1998; Nebauer et al., 1999) and phylogenetic studies (Hess et al., 2000). Data produced with ISSR and other method of PCR finger printing may be analyzed for phylogenetic purposes but are more commonly used for isolates.

PCR finger printing is recommended as a basic tool for proving the identity of strains, especially with regard to comprehensive culture collections.

Dendrogram generated by ISSR primers

Nine inter simple sequence repeat (ISSR) primers were screened on 45 isolates for genotypic finger printing. The isolates shows clearly scorable polymorphism and similarity coefficient ranged from 0.00 to 0.44. A perusal of dendrogram (Figure 1) indicates that there was a major cluster consisting of 44 out of 45 isolates. The major cluster A and minor cluster B consisted of 44 and 1 isolates (P_{224}). The major cluster A showed sub-clustering near 5% similarity level with A_1 consisting of 43 isolates and A_2 consisting of 1 isolate (P_{223}). The cluster A_1 further showed sub-clustering near 0.12 similarity levels as sub-cluster $A_{1(a)}$ and $A_{1(b)}$ and consisted of 41 and 2 isolates, respectively. Cluster $A_{1(a)}$ further showed sub-clustering near 0.14 similarity levels as sub-cluster $A_{1(a1)}$ and $A_{1(a2)}$ and consisted of 38 and 3 isolates, respectively. Cluster $A_{1\ (a1)}$ further sub-clustered at nearly 0.15 similarity level. Cluster $A_{1(a1)}$ sub-clustered as $A_{1(a1\text{-}1)}$ and $A_{1(a1\text{-}2)}$ which consisted of 37 and 1 isolates, respectively. The cluster $A_{1(b)}$ consisting of 2 isolates showed sub-clustering near 0.14 similarity level as $A_{1(b1)}$ and $A_{1(b2)}$ that corresponds to the isolates (P_{113}) and (P_{120}). Cluster B consists of only one isolates (P_{224}) and was genetically dissimilar with all 44 isolates. All the isolates are variable and formed similarity groups in subsequent sub-sub- clustering at approximately 0.12 to 0.33 similarity coefficient. No correspondence was observed between the ISSR based clustering and pyocinin and fluorescine expression.

Individual cluster grouped different types of isolates irrespective of their expression for pyocyanin, fluorescein or siderophore production and are detailed as follows:

Cluster I: P6, P18, P19, P20 and P21 were medium pyocyanin producing and P6, P14, P62 and P76 were high fluorescein producing isolates.
Cluster II: High siderophore producing isolates P9, P21, P167, P109, P144, P133 and P195.
Cluster III: Medium to high fluorescein producing isolates P28, P136, P29, P178, P226, P153, P127, P155, P196, P201 and P221.
Cluster IV: Medium to high fluorescein producing isolates P16, P57, P98, P59, P138, P85, P179, P184 and P82.
Cluster V: High in siderophore producing isolates P52, P70, P123 and P113.

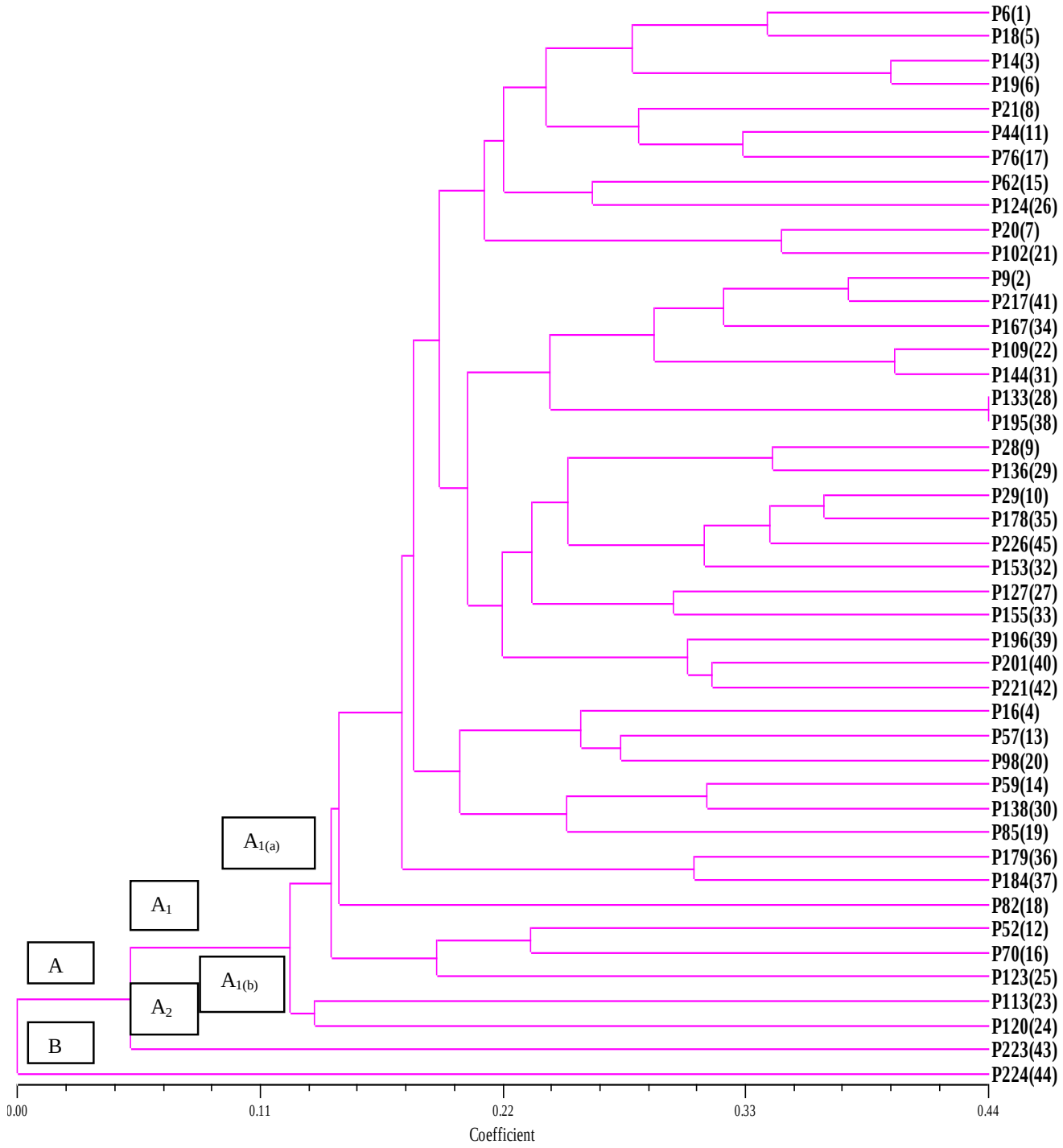

Figure 1. Dendrogram constructed by UPGMA of the binary matrix obtained from ISSR primer based DNA finger printing of 45 isolates of fluorescent *Pseudomonas* using Jaccard (1912) similarity coefficient.

Dendrogram generated by Plt (gene specific primer) based DNA finger printing

Four set of gene specific primer were screened on 45 isolates to identify the primer that produce the polymorphic fragment. The isolates shows clearly scorable polymorphism and similarity coefficient ranged from 0.01 to 0.83. A perusal of dendrogram (Figure 2) indicates that there was a major cluster consisting of 44 out of 45 isolates. The major cluster A and B consisted of 44 and 1 isolates (P_{133}) and shared 0.01 similarity coefficient. The major cluster A showed sub-clustering near 6% similarity

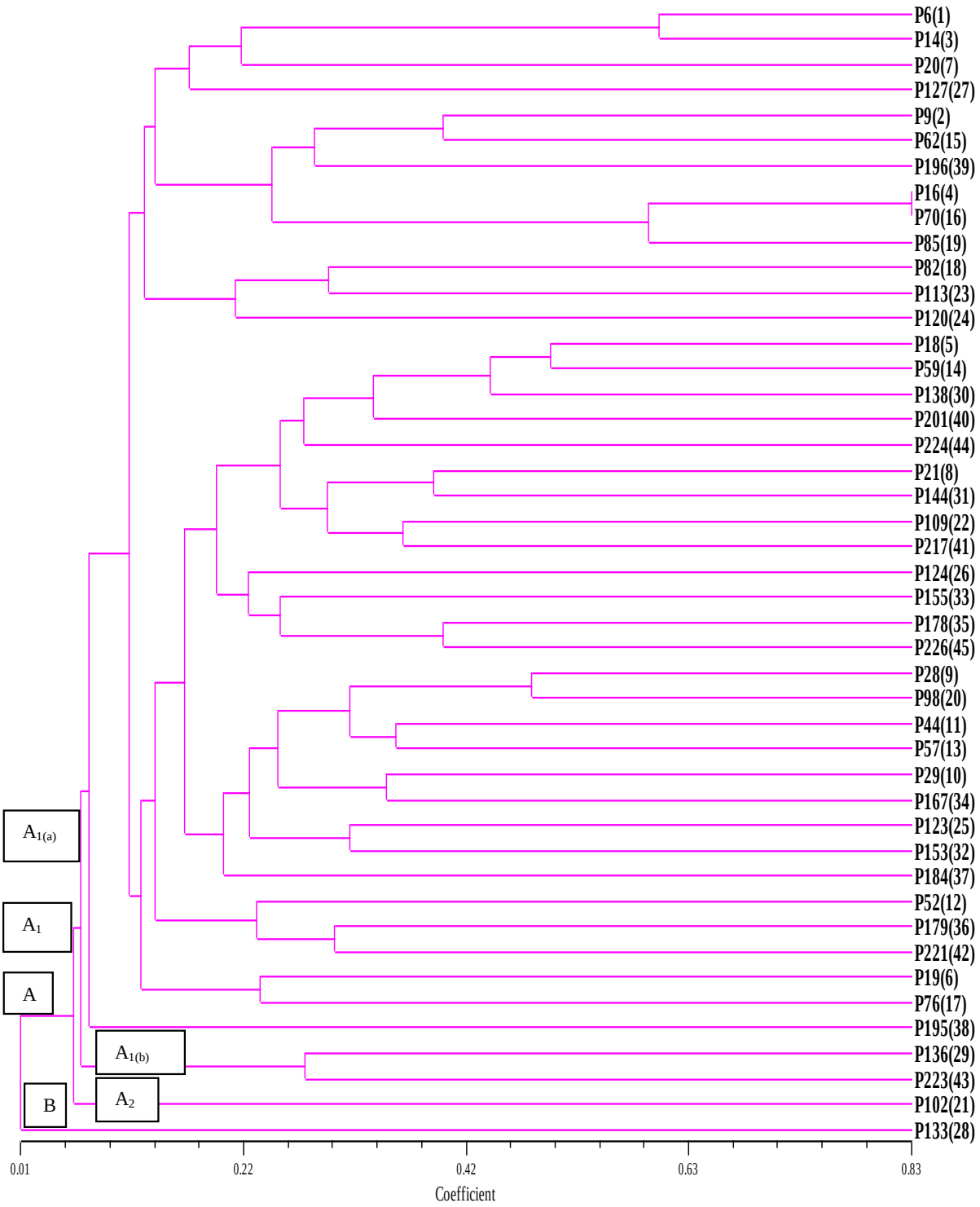

Figure 2. Dendrogram constructed by UPGMA of the binary matrix obtained from gene specific primer (Plt) based DNA finger printing of 45 isolates of fluorescent *Pseudomonas* using Jaccard (1912) similarity coefficient.

level with A_1 consisting of 43 isolates and A_2 consisting of 1 isolates (P_{102}). The cluster A_1 further showed sub-clustering near 0.07 similarity levels as sub-cluster $A_{1(a)}$ and $A_{1(b)}$ and consisted of 41 and 2 isolates, respectively. Cluster A_{1a} further showed sub-clustering near 0.08 similarity levels as sub-cluster $A_{1(a1)}$ and $A_{1(a2)}$ and consisted of 40 and 1 isolates, respectively. Cluster $A_{1(a1)}$ further sub-clustered at nearly 0.12 similarity level. Cluster $A_{1(a1)}$

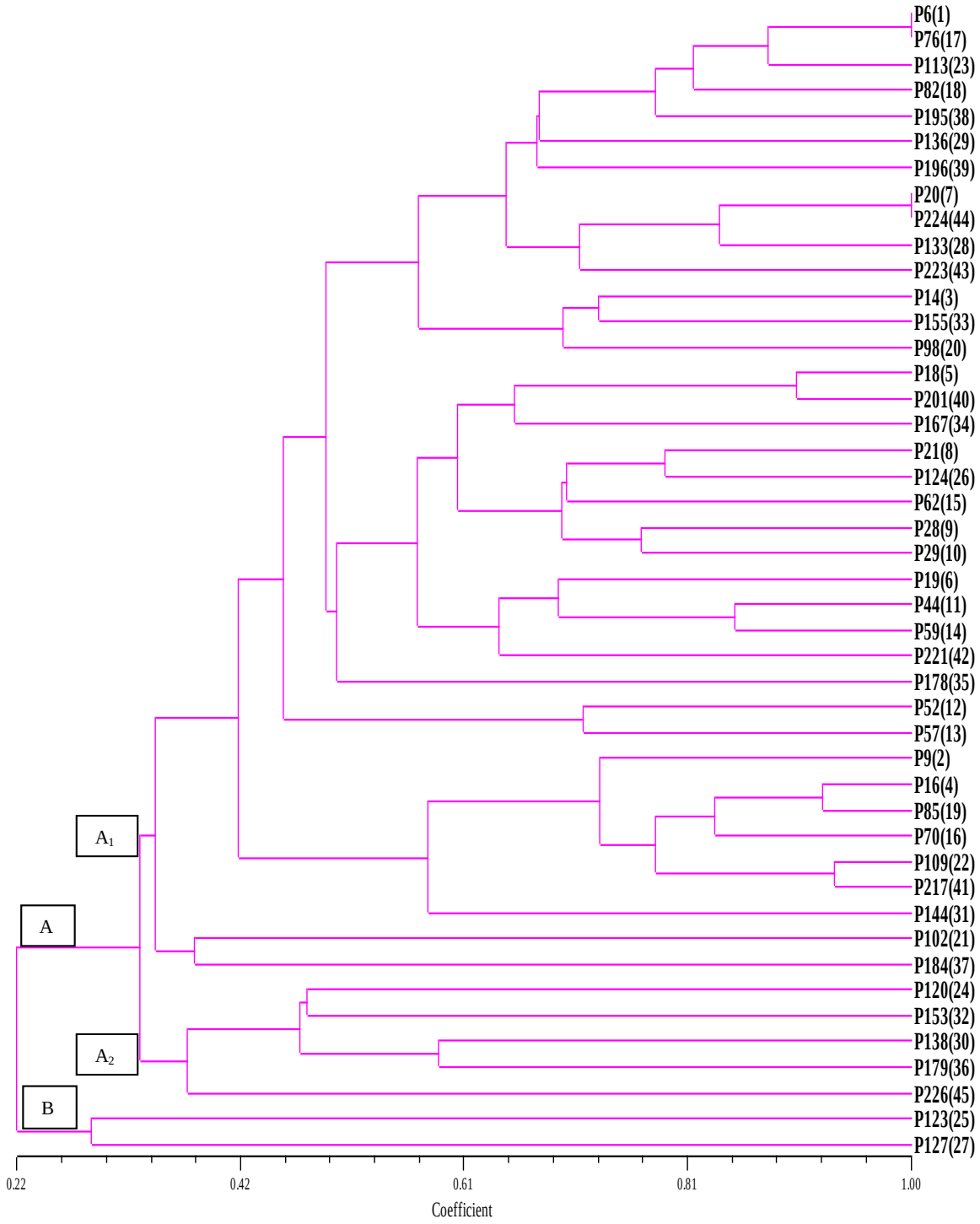

Figure 3. Dendrogram constructed by UPGMA of the binary matrix obtained from 16S rRNA derived primer (AF & EF) based DNA finger printing of 45 isolates of fluorescent *Pseudomonas* using Jaccard (1912) similarity coefficient.

sub-clustered as $A_{1(a1\text{-}1)}$ and $A_{1(a1\text{-}2)}$ which consisted of 12 and 28 isolates, respectively. The cluster $A_{1(b)}$ consisting of 2 isolates showed sub-clustering near 0.28 similarity level as $A_{1(b1)}$ and $A_{1(b2)}$ that corresponds to the isolates (P_{136}) and (P_{223}). Cluster B consists of only one isolates (P_{133}) and genetically dissimilar with all 44 isolates. All the isolates are variable and only formed similarity groups in subsequent sub-sub- clustering at approximately 0.14 to 0.42 similarity coefficient. P6, P14, P20, P127, P9, P62, P16, P196, P70, P85, P82, P113 and P120 clustered together showing nearly 0.11 to 0.58 similarity coefficient.

In second cluster, the isolates P18, P59, P138, P201,

P224, P21, P144, P109, P217, P124, P155, P178 and P226 were showing similarity coffecient of 0.20 to 0.52. The genetically similar isolates P28, P44, P98, P57, P29, P167, P123, P153 and P184 clustered together showing 0.21 to 0.50 similarity coffecient.

Dendrogram generated by primers from 16S ribosomal RNA

Two set of 16S ribosomal RNA specific primer were screened on 45 isolates to identify the primer that produce the polymorphic fragment. The isolates shows clearly scorable polymorphism and similarity coefficient ranged from 0.22 to 1.00. A perusal of dendrogram (Figure 3) indicates that there was a major cluster consisting of 43 out of 45 isolates. The major cluster A and B consisted of 43 and 2 isolates and shared 0.22 similarity coefficient. The major cluster A showed sub-clustering near 32% similarity level with A_1 consisting of 38 isolates and A_2 consisting of 5 isolates. The cluster A_1 further showed sub-clustering near 0.34 similarity levels as sub-cluster $A_{1(a)}$ and $A_{1(b)}$ and consisted of 36 and 2 isolates, respectively. Cluster A_{1a} further showed sub-clustering near 0.41 similarity levels as sub-cluster $A_{1(a1)}$ and $A_{1(a2)}$ and consisted of 29 and 7 isolates, respectively. Cluster $A_{1(a1)}$ further sub-clustered at nearly 0.46 similarity level. Cluster $A_{1(a1)}$ sub-clustered as $A_{1(a1-1)}$ and $A_{1(a1-2)}$ which consisted of 27 and 2 isolates, respectively. Cluster B sub-clustered near 0.29 similarity coefficient as B_1 (P_{123}) and B_2 (P_{127}). All the isolates are variable and only formed similarity groups in subsequent sub-sub-clustering at approximately 0.38 to 0.81 similarity coffecient.

The isolates P6, P76, P113, P82, P195, P136, P196, P20, P224, P133, P223, P14, P155 and P98 were clustered together on the dendrogram and showed 0.57 to 0.88 similarity coefficients. The isolates P18, P201, P167 P21, P124, P62, P28, P29, P19, P44, P59, P221 and P178 were making a cluster group sharing similarity coefficient of 0.52 to 0.87. Another cluster group of the isolates P9, P16, P85, P70, P109, P217 and P144 were showing similarity coefficients of 0.58 to 0.98 signifying genetically much similar. The cluster groups of P16, P57, P98, P59, P138, P85, P179 and P184 more diverse sharing only 0.37 to 0.57 similarity coefficient. Phylogenetic comparisons based on either sequencing or amplified ribosomal DNA restriction analysis (ARDRA) of 16S ribosomal DNA genes have revealed three distinct lineages (phylogenetic-groups) among 45 DAPG + and 138 DAPG + fluorescent *Pseudomonas* spp. Results of ARDRA indicate that these lineages do not correlate with the geographical origin of isolates, which were obtained from several crop species and continents (Keel et al., 1996; McSpadden Gardener et al., 2000). An additional degree of variation among isolates has been observed by using finer scale genotyping based on genomic fingerprinting by randomly amplified polymorphic DNA (RAPD) (Keel et al., 1996; Picard et al., 2000; Raaijmakers and Weller, 2001) and rep-PCR (McSpadden Gardener et al., 2000; Landa et al., 2002). Ten genes (*plt*) required for the biosynthesis of pyoluteorin, an antifungal compound composed of a bichlorinated pyrrole linked to a resorcinol moiety were identified within a 24-kb genomic region of *Pseudomonas fluorescens* Pf-5. They were identified previously by Tn*5* mutagenesis and cosmid cloning (Kraus and Loper, 1995).

Primers PrnCf and PrnCr for the pyrrolnitrin (Prn) biosynthetic locus were developed from sequences within *prn*C (GenBank accession no. U74493) of *P. fluorescens* BL915, which encodes a halogenase that catalyzes chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (Kirner, 1998). Number of PCR product was amplified from any of the other *phlD* strains. The gene-specific primers Phl2a (5'-GAGGACGTCGAAGACCACCA-3') and Phl2b (5'-ACCGCAGCATCGTGTATGAG-3') were used to amplify a 745-bp fragment of *phlD.* For most strains, the cycling program was as described by Raaijmakers et al. (1997). PCR based DNA finger printings revealed polymorphism between 45 isolates of *Pseudomonas* spp. with no correspondence to biochemical tests or pigment production.

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