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Assessment of Genetic Diversity in *Pseudomonas fluorescens* using PCR-based Methods

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ABSTRACT

Biological control of crop diseases and pests using microbial inoculants is being increasingly recognized as a viable, eco-friendly alternative that limits the use of chemical pesticides. A variety of microorganisms inhabit the rhizosphere, and among those certain strains of fluorescent pseudomonads have received much attention because of their potential to function as biological agents for the control of soil-borne pathogenic fungi that cause a number of diseases in crop plants. A number of strains of *Pseudomonas fluorescens* were isolated from the soil rhizosphere and other sources with a view to use them as biological control agents for different crops. The genetic diversity of *P. fluorescens* strains was assessed by two PCR-based molecular techniques, RAPDs and Rep-PCR. Both methods effectively assessed the diversity of the 15 *P. fluorescens* isolates. Though clustering of the various isolates into different groups was not similar, the uniqueness one of the isolates, Pf-6, was demonstrated by the formation of a separate group in both methods. Rep-PCR appears to be more consistent and reliable for diversity assessment as the PCR primers are targeted to a specific region unlike RAPDs, which uses shorter oligonucleotide primers.

Keywords: biocontrol, RAPDs, Rep-PCR

INTRODUCTION

Biocontrol agents are emerging as an important component of integrated pest management (IPM) practices in many of the crops. Biological control of diseases and pests of crops using microbial inoculants is receiving increased attention as an environmentally friendly alternative to the use of chemical pesticides (Fenton *et al*. 1992; Cook *et al*. 1996; Sharifi-Tehrani *et al*. 1998). Exploitation of beneficial plant-microbe interactions in the rhizosphere can result in the promotion of plant health and have significant implications for low input sustainable agriculture applications such as biocontrol. Bacteria such as *Bacillus* and *Pseudomonas*, and fungi such as *Trichoderma*, have been developed as commercial biocontrol products (Keel *et al*. 1996; Haas and Défago 2005).

A variety of microorganisms inhabit the rhizosphere, and among those certain strains of fluorescent pseudomonads have received particular attention because of their potential to function as biological agents for the control of soil-borne pathogenic fungi and oomycetes that attack plants roots and cause considerable damage to crops worldwide (Raaijmakers *et al*. 1997; Gardener *et al*. 2000; Landa *et al*. 2002; de Souza *et al*. 2003; Ramette e*t al*. 2003; Weller *et al*. 2007). A wide range of important fungal diseases in different crops such as root-rot of pea, tobacco and wheat, some root-associated fluorescent *Pseudomonads* produce and excrete secondary metabolites which are inhibitory to plant-pathogenic rhizosphere inhabitants, including fungi, bacteria, and nematodes (Haas and Keel 2003). The understanding of these molecular signaling processes and the functions they regulate is fundamental to promoting beneficial microbe-plant interactions, to overcome existing limitations and to designing improved strategies for the development of novel *Pseudomonas* biocontrol inoculants consortia (Mark *et al*. 2006). More recently, the development of molecular techniques has yielded innovative alternative tools for understanding and demonstrating the mechanisms underlying biocontrol properties (Massart and Jijakli 2007).

The production of antimicrobial secondary metabolites often represents a key factor in their ability to protect plant roots from fungal soil-borne diseases (Keel *et al*. 1992; Dowling *et al*. 1994; Sarniguet *et al*. 1995). It is important to identify the pathogens prevalent in the soil for the crop concerned and identify the enemies that will kill the pathogen (Ramette *et al*. 2006). There are many ways of enriching the soil microflora with the invasive and beneficial bacteria or fungi which will colonize on the pathogens in question or release chemical components that will not allow the pathogens to multiply. Many plant growth-promoting rhizobacteria (PGPR) which support plant growth in different ways. First, they prevent the multiplication of phytopathogens by secreting some chemicals in the soil and also produce certain other chemical substances that stimulate plant growth by making available certain nutrients like Fe, P, etc. in the plant rhizosphere.

The root-colonizing fluorescent pseudomonads produce a diversity of metabolites extracellularly with antimicrobial activity, some of which have a determinative role in disease suppression (Thomashow and Weller 1995; Haas and Keel 2003: Haas and Défago 2005). Antibiotic compounds produced by fluorescent *Pseudomonas* strains play key a role in the suppression of various soil-borne plant pathogens (Schnider *et al*. 1995; Thomashow *et al*. 1995; Sharifi-Therani *et al*. 1998). Some PGPR can also produce enzymes that can lyse fungal cells. For example, *P. stutzeri* produces extra cellular chitinase and laminarinase, which could lyse the mycelia of *F. solani*. Some other PGPR synthesize antifungal antibodies, e.g. *P. fluorescens* produces 2, 4-diacetyl phloroglucinol, which inhibits growth of fungi (Nowak-Thompson *et al*. 1994).

The important antibiotic compounds for which a major contribution to biocontrol has been demonstrated include 2,

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4-diacetylphloroglucinol (DAPG), pyoluteorin, phenazines, pyrrolnitrin, cyclic lipopeptides, and hydrogen cyanide (Haas and Keel 2003; Haas and Défago 2005). In general, effective biocontrol pseudomonads produce at least one of these diffusible or volatile antibiotics. Some strains, such as *P. fluorescens* HA0 and Pf-5, produce multiple antibiotics with overlapping or different degrees of activity against specific pathogens (Haas and Défago 2005).

The diversity of pseudomonads present in the soil also depends on the crop in the field and the type of metabolites that produce to arrest of the pathogens. Further differentiation within the same class of pseudomonads based on the chemical component can be done with the help of molecular markers. Many molecular methods are used to detect the presence of soil-borne pathogens and also to assess the genetic variability among the different isolates (Mavrodi *et al*. 2001; Kumar *et al*. 2002). Application of molecular marker techniques has been useful for studying the genetic changes in the pathogen populations. Promising biocontrol pseudomonads may be identified functionally based on amplified ribosomal DNA restriction analysis (ARDRA) fingerprints (O'Sullivan *et al*. 1992; Gardener *et al*. 2000; Guo *et al*. 2007; Tran *et al*. 2008). In another study, Picard *et al*. (2000) differentiated 64 genotypes based on random amplified polymorphic DNA (RAPD) from a single ARDRA group of phlD-containing isolates from the roots and rhizosphere of maize. Of the several molecular markers, amplified fragment length polymorphism (AFLP) has been found to be a better method for detecting genetic variability among fungal pathogens (O'Neill *et al.* 1997). Gardener *et al*. (2000) identified 13 and 15 genotypes by BOX-PCR and Enterobacterial repetitive intergeneric consensus (ERIC)-PCR, respectively, in a collection of phlD-containing strains. A pair of sequence characterized amplified region (SCAR) primers specific to *P. fluorescens* Pf29A was described by Chapon *et al.* (2003).

A method referred to as rep-polymerase chain reaction (rep-PCR) is a specific bacterial genomic fingerprinting technique that is extremely reliable, reproducible, rapid and highly discriminatory for differentiation of bacterial isolates species, subspecies and strain level. When primers designed against these sequences are used in PCR, the reactions are termed Rep-PCR, Eric-PCR and Box-PCR and collectively they are referred to as Rep-PCR. Fractionation of the PCR products yields a complex fingerprinting pattern with which the bacterial isolates could be differentiated (Rademaker and de Bruijn 1997). Rep-PCR fingerprinting makes use of DNA primers complementary to naturally occurring highly conserved DNA sequences, present in multiple copies in the genomes of most Gram–negative and several Gram–positive bacteria.

In the present investigation, 15 field and commercial isolates of *P. fluorescens* from fields having major legume and oilseed crops of research interest were genetically characterized using two PCR-based molecular methods, i.e. RAPD and rep-PCR. The various isolates were grouped based on the polymorphic pattern obtained with the two techniques.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this project were of molecular biology grade. Tris, CTAB, Proteinase K and Agarose were obtained from Sigma Chemical Co. (USA), dNTPs from GE Healthcare (USA) and *Taq Polymerase* from Genei (Bangalore, India). Standard solutions and buffers were prepared according to the procedures given by Ausubel *et al*. (1999).

Bacterial strains

The different *P. fluorescens* isolates were collected from various agricultural research stations of ANGRAU growing on different crop plants (**Table 1**). The strains were purified as described by Johnson and Curl (1972). Isolates Pf-1 to Pf-11 were obtained from Shamshabad, Ranga Reddy District, Pf-12 from Agnee Co., Hyderabad, Pf13 from the Directorate of Biological Control, Bangalore, Pf-14 from Sree Ram Biotech, Hyderabad and Pf-15 from Directorate of Oilseeds Research, Rajendranagar.

Table 1 Source of *Pseudomonas fluorescens* used.

P. fluorescens strain	Crop grown
Pf-1-3	Tomato
Pf-4-6	Brinjal
Pf-7-9	Chilli
Pf-10,11,15	Sunflower
Pf-12-14	Multiple crops

Bacterial genomic DNA isolation

Genomic DNA was isolated following the method of Ausubel *et al*. (1999). Six ml of bacterial culture grown overnight for 16-18 hr was used for isolation. The culture was centrifuged in a 1.5 ml Eppendorf tube for 2 min at 10,000 rpm. The pellet was resuspended in 0.567 ml of TE buffer by repeated pipetting. To this suspension 30 µl of 10% SDS and 3 µl *Proteinase* K (20 mg/ml) were added, mixed well and incubated at 37°C for 1 h followed by addition of 100 µl of 5 M NaCl. To this mixture 80 µl of CTAB (10%) and NaCl (0.7 M) solution was added, mixed well and incubated at 65°C for 10 min. Equal vol of phenol/chloroform was added, gently mixed and centrifuged at 12,000 rpm for 7 min. The upper phase was taken to a fresh 1.5 ml tube, equal volume of chloroform: isoamyl alcohol (24: 1) was added, mixed well and centrifuged at 12,000 rpm for 5 min. This step was repeated twice. Finally, the supernatant was taken in a fresh 1.5 ml tube and the DNA was precipitated with 0.6 vol of isopropanol. After incubation at room temperature for 30 min, it was centrifuged at 12,000 rpm for 7 min to pellet the DNA. The supernatant was decanted. The pellet was washed with 70% alcohol twice, air-dried and dissolved in TE [10 mM Tris-HCl, 1 mM EDTA, pH 8.0].

DNA samples were quantified by running on agarose gels along with standard DNA and staining with ethidium bromide. Samples were mixed with appropriate amount of 6X loading dye and electrophoreses on 0.8% agarose gel along with varying concentrations of λ DNA (New England Biolabs, USA). The ethidium bromide stained gels were placed on a UV transilluminator (Syngene, USA) and visual comparisons were made with the standards to estimate the DNA concentration in samples.

Analysis of data for RAPD and Rep-PCR

1. RAPD analysis

RAPD analysis was performed following the method of Williams *et al*. (1990) with necessary modifications. A total number of 40 primers (OPA and OPC) supplied by Operon Technologies, USA were used in this study. Genomic DNA (25-50 ng/µl) of the *P. fluorescens* isolated was used as template and PCR amplification was performed in a 20 µl reaction mixture containing 2 µl template, 2 µl of 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 µl (10 ρmol) RAPD primer, 1 U *Taq Polymerase* (Genei, Bangalore). PCR reaction was carried out in a DNA thermocycler (Eppendorf, Germany) with a heated lid. The amplification conditions were as follows: Initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 37°C for 45 sec and extension at 72°C for 1 min. A final extension at 72°C was carried out for 8 min. After PCR, the samples were loaded onto a 1.5% agarose gel along with standard markers of 100 bp and 1 Kb ladders (New England Biolabs, USA).

2. Rep-PCR analysis

In rep-PCR three families of repetitive sequences have been used, including the repetitive extragenic palindromic (REP) sequence, enterobacterial repetitive intergenic consensus (ERIC) and BOX element elements (Lupski *et al*. 1992). Rep-PCR fingerprinting is

Table 2 Primers used in Rep-PCR.

Primer	Sequence (5′-3′)	Number of bases	Recommended annealing temp (°C)
BOXA1 R	CTACGGCAAGGCGACGCTGACG	22	52
ERIC R	TGTAAGCTCCTGGGGATTCAC	21	53
ERIC F	AAGTAAGTGACTGGGGTGAGCG	22	53
REP R	IIIICGICGICATCIGGC	18	46
REP F	ICGICTTATCIGGCCTAC	18	46

a highly reproducible and simple method to distinguish closely related microbial strains, to deduce phylogenetic relationships and to study their diversity in different ecosystems (de Bruijn *et al*. 1992). Three families of repetitive sequences have been studied in most detail, including the 35-40 bp repetitive REP sequence, thee 124-127 bp ERIC sequence and the 154 Box element (Versalovic *et al.* 1994). The corresponding protocols are collectively referred to as rep-PCR. The primers are given in **Table 2** where I is Inosine and were modified from those of de Bruijn *et al*. (1992). Primers ERIC R and ERIC F were as described by de Bruijn *et al*. (1992). Primer BoxA1R was as described by Louws *et al*. (1994). PCR amplification was performed based on Lupski *et al*. (1992) in a 20 µl reaction mixture containing 2 µl template, 2 µl of 10X PCR buffer (Genei, Bangalore), 2 mM $MgCl_2$, 0.2 mM dNTPs (GE Healthcare, USA), 1 µl (10 ρmol) of each forward and reverse primer and 1 U of *Taq Polymerase* and 25-50 ng of bacterial genomic DNA. PCR was performed in a Master Cycler (Eppendorf, Germany). The amplification conditions were as follows: Initial denaturation at 94°C for 3 min followed by 45 cycles of denaturation at 94°C for 25 sec, annealing at 52°C for Box A1R and ERIC primers Box A1R and ERIC primers for 1 min and at 38°C for Rep primers, and extension at 72°C for 1 min. A final extension at 72°C was carried out for 8 min. To confirm the repeatability of the results, these PCR experiments were repeated at least three times with two sets of independently isolated bacterial genomic DNA of each bacterial isolate. The amplicons were visualized under UV light after staining with ethidium bromide.

Data analysis

The experiments were repeated a minimum of three times to confirm the banding pattern and only those consistent bands on the gels were scored for data analysis. The gels were scored for the presence (1) or absence (0) of the corresponding band in the different local isolates. A score of '1' was given for the presence and '0' for the absence of bands. The binary data generated was analyzed for genetic similarity using unweighted pair group arithmetic mean (UPGMA) program of NTSYSpc software, v. 2.11. The dendrograms obtained served as the basis for assessing the genetic relatedness of the *P. fluorescens* strains within the species.

RESULTS AND DISCUSSION

In the present work a total of 40 oligonucleotide primers

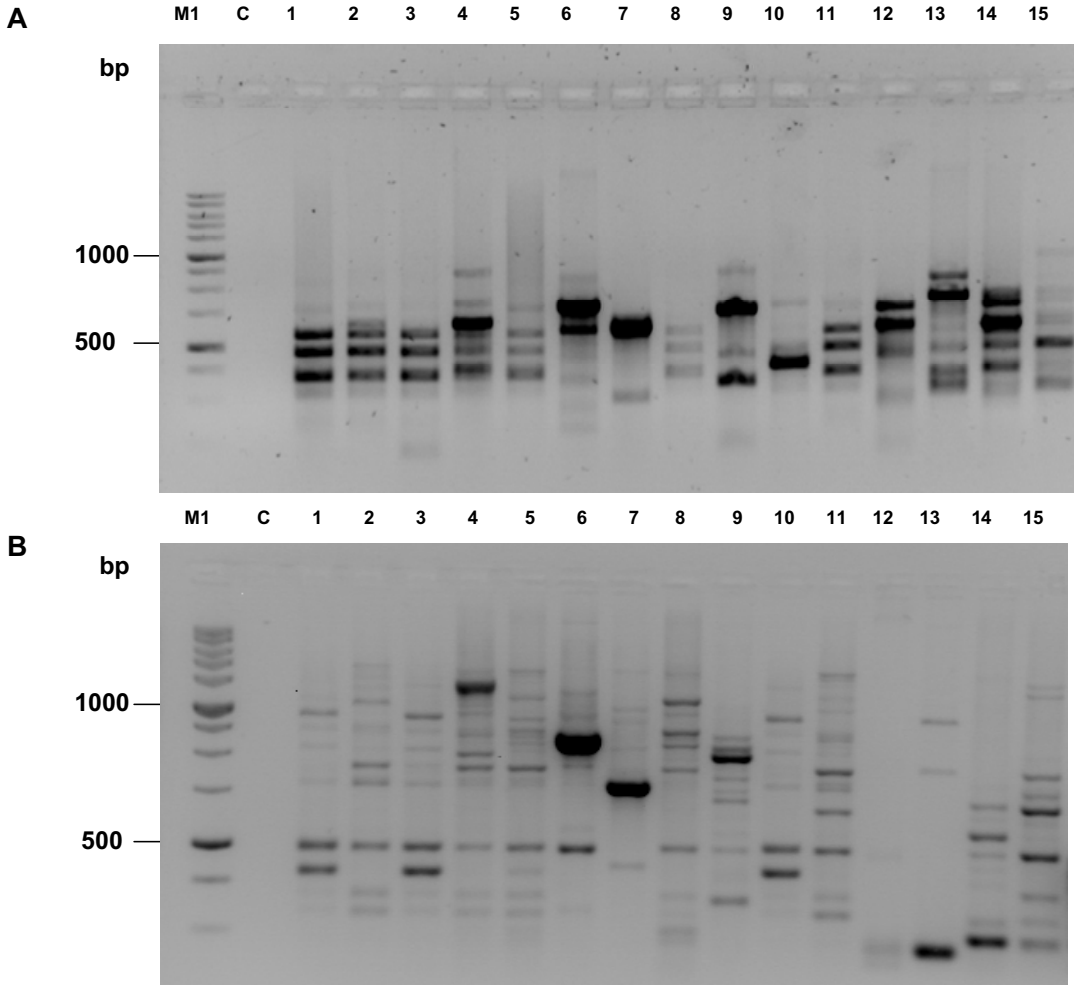

Fig. 1 Agarose gel electrophoresis pattern of *P. fluorescens* DNA amplified with oligonucleotide primers in RAPDs. (**A**) OPA-7 ad (**B**) OPA18. PCR was carried out as described in the Materials and Methods and the amplicons were separated on 1% agarose gel in TAE buffer and viewed after ethidium bromide staining on a transilluminator. Lanes 1-15: Bacterial isolates Pf-1 to Pf-15 as indicated in **Table 1**. Lane M1; 1 Kb DNA ladder. Lane C: PCR control reaction with no DNA template.

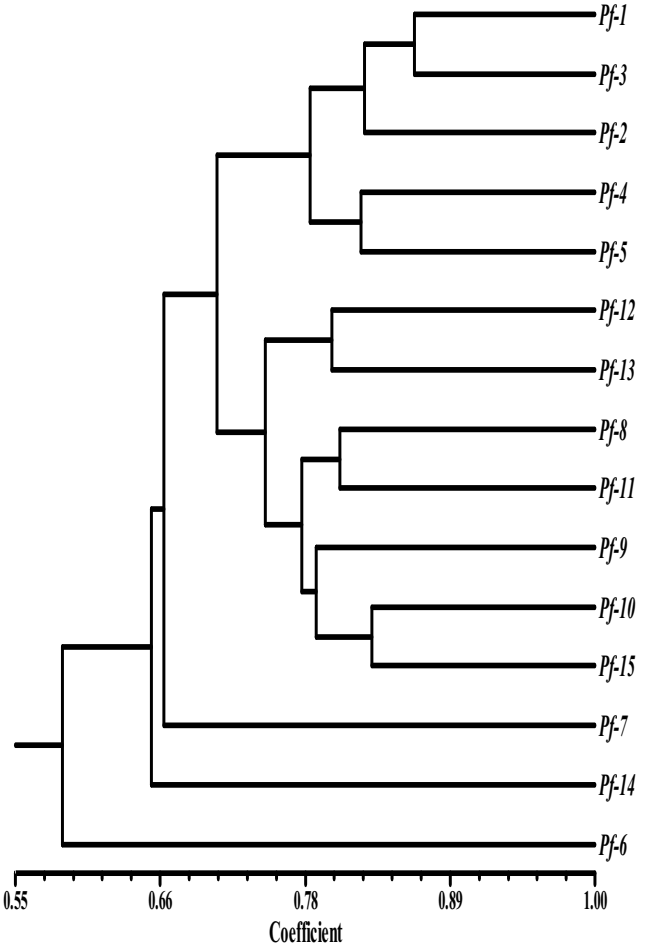

Fig. 2 Dendrogram based on similarity index data derived from the RAPD marker analysis on bacterial isolates Pf-1 to Pf-15. The dendrogram was constructed based on the RAPD marker data obtained with the 15 *P. fluorescens* isolates by UPGMA method as described in the Materials and Methods.

were used in RAPDs and 26 were found to be polymorphic. A representative agarose gel pattern obtained on RAPDs with two oligonucleotide primers is shown (**Fig. 1A, 1B**). The number of DNA bands amplified varied from 2-10 with each primer and the level of polymorphism was mostly 100% in all cases. The amplified DNA fragments ranged from 300-6,500 bp. The number of polymorphic loci amplified varied with the different primers and isolates.

All the bands were scored for their presence and absence in the 15 bacterial isolates and a similarity matrix was constructed using the UPGMA program. Cluster analysis carried out based on the similarity data generated from the 15 isolates using the 26 primers accounted for a total of 160 polymorphic DNA bands. The various *P. fluorescens* isolates were divided into 5 major classes (**Fig. 2**). Three of the isolates i.e. Pf-6, Pf-14 and Pf-7 formed three separate groups by themselves. The other two major classes consisted of different subgroups with Pf-7 and Pf-5 isolates, respectively. The similarity among these standard isolates ranged from 0.42 to 0.95.

A high level of polymorphism was seen in PCR with the REP, ERIC, and BOX set of primers (**Fig. 3A-C**). The number of DNA bands generated by PCR with the above-mentioned three primers varied in size from 300-6,500 bp and also in the total number of bands seen. The total number of polymorphic DNA bands seen in this method was almost similar to that observed with RAPDs. The percentage polymorphism with the three primers was 100% with REP and BOX primers but only 86% with the ERIC primer. A few of the bacterial isolates showed unique bands indicating the ability of Rep-PCR to distinguish many of the isolates.

All the bands were scored for their presence and absence in the 15 bacterial isolates and a similarity matrix was constructed using the UPGMA program. The dendrogram obtained from the combined data from all three primer sets indicated that the similarity among these standard isolates ranged from 0.42 to 0.95 (**Fig. 4**). Three major groups were seen with the 15 isolates and Group I contained Pf-12 and Pf-6, Group II Pf-13 and Group III 12 different isolates in a number of subgroups.

Biological control of plant pathogens has stimulated much interest in recent years with the growing trend in agriculture towards greater sustainability and public concern of the use of hazardous pesticides. There is now unequivocal evidence that toxic chemicals and antibiotics play a key role in the suppression of various soil-borne plant pathogens by antagonistic microorganisms. Among the variety of microorganisms inhabiting the rhizosphere, certain strains of fluorescent *Pseudomonads* have received special attention due to their potential as biological agents for the control of soil-borne pathogenic fungi and oomycetes that attack crop plants (Keel and Défago 1996; Haas and Défago 2005: Weller 2007). The fluorescent *Pseudomonads* colonize the roots and produce a diversity of extracellular metabolites with antimicrobial activity, some of which have a specific role in disease suppression (Thomashow and Weller 1995; Haas and Keel 2003). Genetic variability exists among the various isolates of *Pseudomonads* depending on the geographical locations from where these were collected, crops grown in a specific region and the agricultural practices employed in the location (Picard and Bosco 2008).

DNA-based (genotypic) approaches (Saharan and Naef 2008; Schütte *et al*. 2008) have increasingly been applied to microbial identification and classification. In fact, these molecular approaches have resulted in the birth of a new ecology subspecialty. Generally, these methods tend to be dependent on bacterial growth variables, more stable, less time-consuming and are very useful for determining phylogenetic relationships among microbial isolates and for assigning strains into specific groups.

Biocontrol agents have been differentiated based on secondary metabolites (Haas and Keel 2003; Haas and Défago 2005; Couillerot 2009) and within subspecies using the latest biotechnological methods (Manceau and Harvais 1997; Picard *et al.* 2000). Cellular protein profiles have been used to differentiate various *P. fluorescens c*ollected from different crop fields (Shanmugam *et al*. 2008). PCR-based methods such as RAPDs and those using ITS primers have been extensively used (Kumar *et al*. 2002; Walsh *et al.* 2003; Tran *et al*. 2008). The genomic fingerprinting method employed is based on the use of DNA primers corresponding to naturally occurring repetitive elements in bacteria, such as the REP, ERIC and BOX elements, and the PCR reaction (Rep-PCR). Rep-PCR fingerprinting is a highly reproducible and simple method to distinguish closely related strains, to deduce phylogenetic relationships between strains, and to study their diversity in a variety of ecosystems (Versalovic *et al.* 1994; Naik *et al*. 2008a, 2008b).

The similarity data obtained with RAPD primers identified 5 major groups by cluster analysis, three of which had only a single isolate. The grouping does not appear to be based on geographic origin as Pf-6 and Pf-7 were from the same district but not from the same field. The commercial isolates Pf-14 and Pf-12 were in the same group but Pf-15 was grouped with a number of other isolates. The genotypic and phenotypic diversity of *P. flourescens* could be based on the geographic origin or functional differences in the genome of the strains (Naik *et al.* 2008a; Silby *et al*. 2009). RAPDs are able to differentiate a large number of isolates and with the use of more primers it may be possible to get unique bands that can be used for fingerprinting and protecting commercial *P. fluorescens* isolates. It is important to combine molecular diversity with the antibiotic-producing property of the isolates to properly identify the different strains prevalent in crop fields.

The rep-PCR profiles *P. fluorescens* strains indicated that with this technique each set of subspecies could be

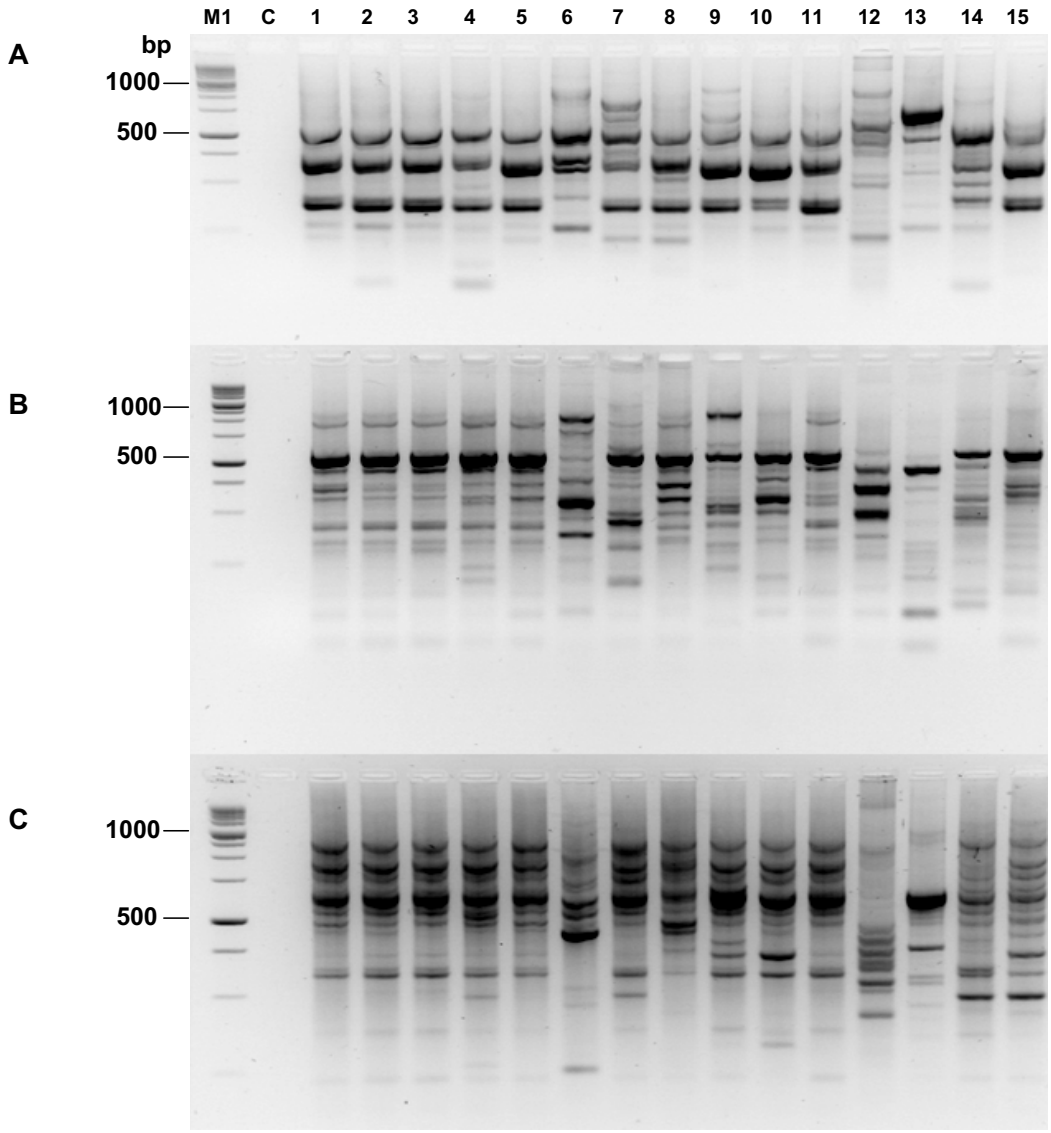

Fig. 3 Agarose gel electrophoresis pattern of *P. fluorescens* DNA amplified with oligonucleotide primers in rep-PCR. (**A**) REP, (**B**) ERIC and (**C**) BOXA1R. PCR was carried out as described in the Materials and Methods and the amplicons were separated on 1% agarose gel in TAE buffer and viewed after ethidium bromide staining on a transilluminator. Lanes 1-15: Bacterial isolates Pf-1 to Pf-15. Lane M1: 1 Kb DNA ladder. Lane C: PCR control reaction with no DNA template.

easily distinguished as they gave unique profiles with each primer set. This technique thus could be used to identify the subspecies to which each of the isolates belonged which was not the case with RAPDs.

The grouping of the 15 isolates obtained by RAPD analysis and Rep-PCR were different. However, isolate Pf-6 was separated from the others in both methods suggesting the uniqueness of this isolate. Though the other two isolates Pf-4 and Pf-5 were isolated from the brinjal growing soil Pf-6 appeared to be unique which only points to the ecological variation among the different fields where other crops could have been grown in different seasons contributing to such differences. Such molecular methods help to identify novel strains of pseudomonads which will be more effective as biocontrol agents in a particular region and for specific pathogens as was reported for black rot of tobacco (*Thielaviopsis basicola*) (Ramette *et al.* 2006). Though both methods could distinguish the 15 isolates, Rep-PCR may be better as it is targeted to a specific region of the genome and is highly reproducible (Binde *et al*. 2009; Palencia *et al*. 2009; Rameshkumar and Nair 2009). Though the studies of Shanmugam *et al*. (2008) reinstated the importance of whole-cell protein analyses in characterizing pseudomonads and assessing their diversity, the isolates representing similar rhizospheres and geographic locations were generally distributed into different phenotypic clusters as influenced by unknown factors. Similarly the clustering in our studies based on DNA also was not representing the variations in the different fields or the crops grown.

By converting the RAPD marker into a SCAR marker it may be possible to increase the reliability of this method although a number of such SCAR primers need to be tested for diversity analysis. It is important to correlate molecular diversity with functional diversity to assess the utility of these isolates as biocontrol agents for a particular crop in local fields which can then be extrapolated to the same crop in other fields or even other crops.

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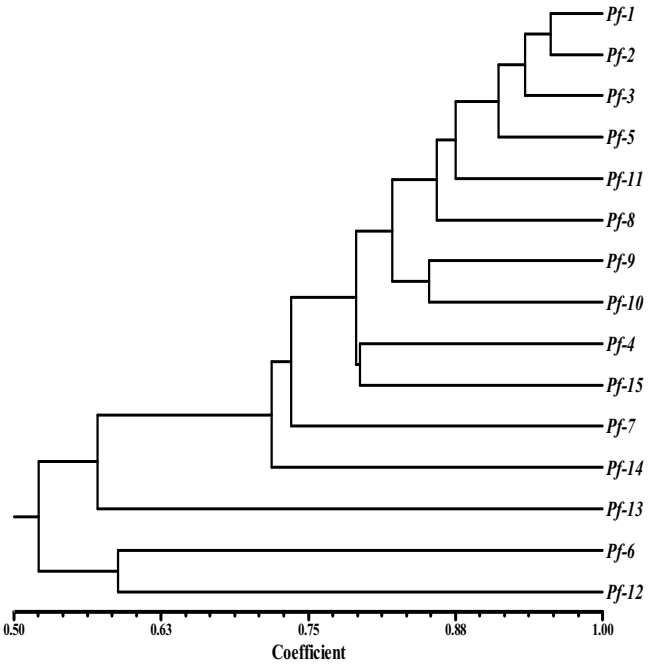

Fig. 4 Dendrogram based on similarity index data derived from the rep-PCR (REP, ERIC and BOX-PCR) marker analysis on bacterial isolates Pf-1 to Pf-15. The dendrogram was constructed based on the rep-PCR data obtained with the 15 *P. fluorescens* isolates by UPGMA method as described in the Materials and Methods.

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