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Genotypic and Phenotypic Diversity of PGPR Fluorescent Pseudomonads Isolated from the Rhizosphere of Sugarcane (*Saccharum officinarum* L.)

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The genetic diversity of plant growth-promoting rhizobacterial (PGPR) fluorescent pseudomonads associated with the sugarcane (*Saccharum officinarum* L.) rhizosphere was analyzed. Selected isolates were screened for plant growth-promoting properties including production of indole acetic acid, phosphate solubilization, denitrification ability, and production of antifungal metabolites. Furthermore, 16S rDNA sequence analysis was performed to identify and differentiate these isolates. Based on 16S rDNA sequence similarity, the isolates were designated as *Pseudomonas plecoglossicida*, *P. fluorescens*, *P. libaniensis*, and *P. aeruginosa*. Differentiation of isolates belonging to the same group was achieved through different genomic DNA fingerprinting techniques, including randomly amplified polymorphic DNA (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and bacterial repetitive BOX elements (BOX) analyses. The genetic diversity observed among the isolates and rep-PCR-generated fingerprinting patterns revealed that PGPR fluorescent pseudomonads are associated with the rhizosphere of sugarcane and that *P. plecoglossicida* is a dominant species. The knowledge obtained herein regarding the genetic and functional diversity of fluorescent pseudomonads associated with the sugarcane rhizosphere is useful for understanding their ecological role and potential utilization in sustainable agriculture.

Keywords: PGPR, fluorescent pseudomonads, repetitive sequences, RAPD, ARDRA, 16S rDNA sequence

Sugarcane has gained status as an important cash crop in India for the obvious reasons of profitability and economic importance as well as for the wider requirement for sugar product(s). Consequently, sustaining and enhancing the growth and yield of sugarcane have become a major focus of research. The growth and performance of sugarcane in the field are adversely affected by a number of biotic and abiotic factors, including a wide range of fungal and bacterial diseases. Chemical applications, cultural practices, and the use of resistant cultivars are routine methods of improving the yield of crop plants. However, resistant cultivars are not available for every disease, and cost-effective cultural practices are not always feasible. Moreover, the available chemical fungicides are often expensive and have adverse effects on human health. Indiscriminate use of these chemicals is known to be hazardous to the environment and is lethal to beneficial rhizosphere bacteria.

It has long been recognized that many naturally occurring rhizosphere bacteria and fungi are antagonistic towards crop pathogens and, as a result, may offer a viable substitute for the use of chemicals. Plant growth-promoting rhizobacteria (PGPR) have shown beneficial effects on plant growth and health, their main modes of action being nitrogen fixation, production of phytohormones and antifungal compounds, and induced systemic resistance [7, 18]. Rhizosphere bacteria are regarded as a large resource for the discovery of isolates with novel antifungal and plant growth-promoting traits.

In this direction, PGPR fluorescent pseudomonads offer attractive alternatives as bioinoculants and biocontrol agents. In order to utilize PGPR pseudomonads successfully as bioinoculants in agriculture, it is important to understand their genotypic diversity and metabolic versatility and their capacity to produce a wide range of antimicrobial metabolites [22]. Fluorescent pseudomonads exhibit diversity with respect to their phenotypic characteristics, and identification of these organisms at the biovar level has been difficult [39]. Conventionally, phenotypic characteristics have been used for the classification of bacteria, but these methods are not precise enough to distinguish between similar organisms

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and to determine their phylogenetic relationships [24]. However, molecular fingerprinting techniques offer an attractive and reliable method of the identifying and cataloging related isolates. The study of phenotypic and genotypic diversity of *Pseudomonas* spp. and their plant growth-promoting potential is important not only for understanding their ecological role in the rhizosphere and their interaction with plants, but also for any biotechnological application [3]. The predominance of fluorescent pseudomonads in rhizosphere soils of plants such as cotton, rice, banana, wheat, and canola has been reported [9, 10, 26, 38]. Several studies have focused on and reported the characterization of endophytic beneficial bacteria associated with sugarcane, and a few have reported on sugarcane rhizosphere-associated bacteria [15, 34]. However, a comprehensive analysis of the diversity and functional activities of fluorescent PGPR pseudomonads associated with the sugarcane rhizosphere has not been reported thus far. Therefore, an extensive evaluation of their distribution, identity, and genetic diversity was attempted in this study. The existence of genetic diversity among PGPR fluorescent pseudomonads isolated from the sugarcane rhizosphere is reported here.

Materials and Methods

Isolation and Screening of PGPR *Pseudomonas*

Fluorescent pseudomonads were isolated from the rhizosphere of sugarcane cultivated in Vagaikulam around Madurai, India. The soil was sand clay loam, with physicochemical properties as follows: soil order, inceptisol; pH, 7.3; EC, 0.08 dS/m; available N, 204 kg/ha; available P, 10.6 kg/ha; available K, 148 kg/ha; organic carbon, 0.65%; and CEC, 22.2 mmol/kg. Roots with adhering soil were homogenized in phosphate-buffered saline solutions, and then serially diluted and plated on King's B medium (*Pseudomonas* isolation agar medium). The plates were incubated at 30°C for 24 h, and colonies that fluoresced under UV light ($\lambda = 356$ nm) were selected and further purified on the same medium.

Functional and Phenotypic Characterization of Fluorescent *Pseudomonads*

Indole acetic acid (IAA) production by the cultures was determined by the method of Patten and Glick [24] For determining denitrification, the culture was inoculated into 10 ml of nitrate broth at room temperature. On days 7, 14, 21, 28, and 35, 2 ml of the broth was tested by adding equal amounts (5 drops) of sulfanilic acid and alpha-naphthylamine. Development of a red color indicated the reduction of nitrate to nitrite. To the broth not showing color change, a microspatula of zinc powder was added. The absence of a red color indicated that denitrification had occurred. The phosphate solubilizing activity of the cultures was determined after growth on Pikovskaya's agar (Hi-Media, Mumbai, India) plates at 30°C for 72 h. Colonies exhibiting a halo zone around them were taken as positive for solubilization of tricalcium phosphate [32]. For determining antifungal activity, agar plugs (4 mm diameter) taken from actively growing fungal cultures, namely, *Fusarium moniliformis* (FM), *Fusarium oxysporum* (FO), and *Rhizoctonia bataticola* (RB) (obtained from Tamil Nadu Agricultural University, Coimbatore, India), were placed on the surface of potato dextrose agar plates. Simultaneously, cultures were streaked 3 cm from the agar plug at the sides toward the edge of the Petri plates. A plate inoculated with fungal agar plugs alone was used as a control. The plates were incubated at 30°C until fungal mycelia completely covered the agar surface in the control plate.

Biochemical tests, such as fluorescence on King's B agar, arginine dihydrolase, oxidase, gelatin hydrolysis, levan production, and growth at 4°C and 42°C were performed according to Bossis *et al*. [4]. An antibiotic sensitivity test was performed using a pure culture of the test organism inoculated on Mueller–Hinton agar. Paper disks impregnated with a standardized concentration of individual antibiotics were added to the plates, which were then incubated at 37°C. The size of the clearing zone was read after 18 h of incubation.

Fatty Acid Methyl Ester (FAME) Analysis

For FAME analysis, bacterial cultures were grown on tryptic soy agar in triplicate and incubated at 28°C for 24 h. Cells (50 mg wet weight) were scraped and suspended in 1 ml of saponification reagent in a screw-cap test tube and vortexed for 10 s. The tube was then suspended in a water bath at 100°C for 25 min, and then cooled to room temperature. Two ml of methylation reagent was added and the tube was vortexed for 10 s, placed in a water bath at 80°C for 10 min, and then rapidly cooled by placing it on ice. Then, 1.25 ml of extraction buffer was added and mixed well for 10 min. The aqueous lower layer was separated and discarded. To the upper organic phase, 3 ml of base wash reagent was added and mixed well for 5 min. The mixture was then centrifuged at 3,000 rpm for 5 min. The upper solvent phase was removed and analyzed by gas–liquid chromatography (Hewlett-Packard 6890; Avondale, USA) using an Ultra 2-HP capillary column (cross-linked 5% phenyl-methyl silicone, 25 m, 0.22 mm; film thickness, 0.33 μm) and hydrogen as the carrier gas. FAME compounds were detected by a flame ionization detector and identified using Microbial Identification Software (Version 4.5, Sherlock aerobe method and TSBA40 Library; MIDI Inc., Newark, DE, USA).

Identification of Fluorescent *Pseudomonas*

The 16S rDNA region was amplified using a specific forward primer (PA, 5'-GCATCCAAAACTACTG-3'; PF, 5'-TTGCTTCTCTTGAG-3') and a reverse primer (CR, 5'-TACCTTGTTACGACTTC-3') for identification of fluorescent *Pseudomonas*. To confirm their identity, primers designed to amplify the region between the 16S and 32S rRNA intervening sequence, ITS1 forward (5'-AAGTCGTAACAAGGTAG-3') and ITS2 reverse (5'-GACCATATATAACCCCAAG-3'), were used. Furthermore, the 16S rDNA region was also amplified using *Pseudomonas* genus-specific 16S rRNA-F (20-mer forward, 5'-GGTCTGAGAGGATGATCAGT-3') [1] and 16S rRNA-R (18-mer reverse, 5'-TTAGGTCCACCTCGCGGC-3') [42] gene primers.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

E. coli 16S rDNA primers, forward from positions 7 to 26 (5'-AGAGTTTGATCCTGGCTCAG-3') [6] and reverse from positions 1,513 to 1,494 (5'-ACGGCTACCTTGTTACGACTT-3') [41], were used to amplify the genomic DNA of the isolates. Amplification was performed in a DNA thermal cycler (DNA Engine; MJ Research, USA). The polymerase chain reaction (50 μl) contained 50 pmol of each primer, 50 ng of genomic DNA, 1× *Taq* DNA polymerase

buffer, 1 U of *Taq* DNA polymerase (Promega, Madison, WI, USA), 0.2 mM of each deoxynucleotide triphosphate, and 1.5 mM $MgCl_2$. An expected amplicon of 1.5 kb was obtained from all of the isolates. These fragments were digested with 15 different restriction enzymes (*Eco*RI, *Bam*HI, *Pvu*II, *Eco*RV, *Hin*dIII, *Hae*III, *Bgl*II, *Nhe*I, *Sph*I, *Pst*I, *Kpn*1, *Xma*I, *Nla*IV, *Alu*I, *Pli*I, and *Dpn*I). The digested products were electrophoresed on 12% (w/v) native acrylamide gels, stained with ethidium bromide, and photographed and analyzed using a gel documentation system (Model 2000, Biorad, USA).

Phylogenetic Analysis

The reference sequences required for comparison were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank). The aligned sequences were then manually checked for gaps in each row and saved in molecular evolutionary genetics analysis (MEGA) format using MEGA v.2.1 software. Pairwise evolutionary distances were computed using the Kimura 2-parameter model [12]. To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method. The bootstrapped dataset was used directly for constructing the phylogenetic tree with the MEGA program or for calculating multiple distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using the neighbor-joining method of Saitou and Nei [33]. All of these analyses were performed using MEGA v.2.1 [11].

RAPD Fingerprinting

Three different primers, namely pgs2 (5'-GTTTCGCTCC-3'), pgs3 (5'-GTAGACCCGT-3'), and pgs4 (5'-AAGAGCCCGT-3'), were used for RAPD analysis. PCR amplification was carried out in a 20 µl reaction mixture containing 2 µl of 10× PCR buffer (with 2.5 mM $MgCl_2$), 2 µl of 2 mM dNTP mixture, 5 µl of each 2 µM primer, 3 U of *Taq* DNA polymerase, 8 µl of water, and 50 ng of template DNA. The following cycle parameters were used: 92°C for 45 s, 28°C for 60 s, and 72°C for 90 s. The total number of cycles was 40, with an initial denaturation step extended to 2 min and a final extension time of 10 min. PCR products were electrophoresed on an agarose gel (2%), stained in ethidium bromide solution (0.5 µg/ml) for 30 min, and photographed and analyzed. All PCR–RAPD reactions were repeated at least three times, and the fingerprints were compared; only those RAPD bands that appeared consistently were evaluated. Calculation of the pairwise coefficient of similarity was based on the presence and absence of bands, and cluster analysis was done by the unweighted pair group method with arithmetic mean (UPGMA). The genomic fingerprints obtained were compared for similarity by visual inspection of band patterns. These patterns were converted to a two-dimensional binary matrix (1 = presence of band; 0 = absence of band). Similarity matrices were calculated with the Dice coefficient [28]. Cluster analysis of similarity matrices was performed by the UPGMA. The goodness of the clustering method was assessed by calculating the cophenetic correlation coefficient (r). Computer-assisted analysis was performed with the NTSYS-pc2 program for Windows [33].

Rep-PCR-Based Genotypic Fingerprinting (REP, ERIC, and BOX) Analysis

Rep-PCR amplification was carried out as previously described [2, 3, 18] in a DNA thermal cycler (DNA Engine; MJ Research, USA) using 50 ng of template DNA and a 2 mM concentration of primers. The PCR amplification program for REP was performed with an initial denaturation at 95°C for 7 min; 30 cycles of 94°C for 1 min, 41.8°C for 1 min, and 65°C for 8 min; and a final extension at 65°C for 15 min and 4°C for 5 min. ERIC PCR amplification was performed with an initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min and 4°C for 5 min. BOX-PCR analysis was performed with an initial denaturation at 95°C for 7 min; 30 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min; and a final extension at 65°C for 15 min and 4°C for 5 min. Ten µl of PCR product was separated on 1.5% (w/v) agarose gels stained with ethidium bromide in 1× TAE. The image of the gels was digitized, and computer-assisted analysis of genomic fingerprints was performed using the BIO-GENE software programme (Version 11.02; Vilber Lourmat, France). Similarity matrices of the whole densitometric curves of the gel tracks were calculated using the Dice coefficient. Cluster analysis of similarity matrices was performed by the UPGMA algorithm.

Nucleotide Sequence Accession Numbers

The accession numbers of the 16S rRNA nucleotide sequences of the 25 isolates submitted to GenBank are shown in Table 2.

RESULTS

Isolation and Screening of PGPR *Pseudomonas*

A total of 376 isolates with characteristic fluorescence were isolated from the sugarcane rhizosphere. All of the isolates were screened for functional properties of PGPR; namely, IAA production, denitrification, phosphate solubilization, and antifungal activity. Among them, 25 isolates that exhibited at least two properties of plant growth promotion were selected and designated S1 to S25 (Table 1).

Functional Characterization of PGPR *Pseudomonas*

Analysis of plant growth promotion properties revealed that all of the isolates produced IAA. Among them, 23 isolates exhibited antifungal activity, 18 isolates solubilized phosphate, and 15 isolates exhibited denitrification activity. Furthermore, all of the isolates were tested for *in vitro* antagonistic activity, and were shown to exhibit antifungal activity with the exception of one strain, S22. Antifungal activity was compared in the presence and absence of iron (data not shown). Eighteen isolates inhibited all three fungal pathogens, three isolates exhibited antagonistic activity toward two fungal pathogens, two isolates inhibited only one fungal pathogen, and two isolates inhibited none of the pathogens in the medium without iron supplementation. The antagonistic bacterial strains induced growth-free inhibition zones ranging from 6 to 28 mm in diameter. On the contrary, only eight isolates showed antifungal activity in iron-supplemented medium; however, three of them (S3, S17, and S20) inhibited all three fungal pathogens, whereas the five remaining isolates inhibited one of the pathogens.

Table 1. PGPR properties of fluorescent *Pseudomonas* isolated from sugarcane rhizosphere.

Strain	Accession number	Denitrification	Phosphate solubilization	IAA (μg/100 ml)	Antifungal activity (mm*)		
					FM	*FO*	*RB*
S1	DQ095890	+	+	120	+	+	+
S2	DQ095891	+	+	90	+	++	+
S3	DQ095892	+	-	40	+	+	+++
S4	DQ095893	+	+	115	++	+	+
S5	DQ095894	-	+	160	+	+	+++
S6	DQ095895	+	+	40	+	+	+
S7	DQ095896	+	+	110	+	+	+
S8	DQ095897	-	+	160	++	+	++
S9	DQ095898	-	+	120	+	+	+
S10	DQ095915	+	+	110	+	++	+
S11	DQ095899	-	+	150	+	+	++
S12	DQ095900	-	+	150	+	+	+
S13	DQ095901	+	-	190	+	+	+
S14	DQ095902	+	-	260	++	+	++
S15	DQ095903	-	-	80	+	+	+++
S16	DQ095904	-	+	100	+	+	+
S17	DQ095905	+	-	100	-	-	-
S18	DQ095906	-	+	80	+	+	+
S19	DQ095907	+	+	40	+	+	+++
S20	DQ095909	+	-	40	+	+	+
S21	DQ095908	+	+	110	+	+	+
S22	DQ095910	-	+	170	-	-	-
S23	DQ095882	+	-	40	+	+++	++
S24	DQ095912	-	+	80	+	+	++
S25	DQ095913	+	+	40	+	+	+

FM: *Fusarium moniliformis*; *FO*: *Fusarium oxysporum*; *RB*: *Rhizoctonia bataticola.*
*+ = 6–10 mm inhibition.
*++ = 11–20 mm inhibition.
*+++ = 21–28 mm inhibition.

These results suggest that the antagonistic activities of the majority of the bacterial isolates are siderophore-mediated (Table 1).

Phenotypic Characterization of PGPR *Pseudomonas*

Biochemical characterization of the sugarcane rhizosphere isolates revealed that all are Gram-negative, rod-shaped, non-spore-forming, motile organisms exhibiting catalase, oxidase, and arginine hydrolysis activities. They produce fluorescent pigments, are able to utilize citrate, but are unable to produce indole. Furthermore, the isolates were examined for sugar utilization and their antibiograms were determined (Table 2).

Based on the above characteristics, the isolates could be identified as *P. fluorescens*. To ascertain further the identity of the isolates, 12 were randomly selected and subjected to FAME analysis. Nine out of 12 had the closest match with *P. putida* biotypes. Among them, three isolates, namely S6, S20, and S24, had identity with *P. putida* biotype B whereas S1, S8, S19, S21, S22, and S23 were identified as *P. putida* biotype A. However, S4, S14, and S18 were found to be close to *P. syringae*.

Analysis of 16S rRNA Gene Sequences

An approximately 1.5 kb DNA fragment was PCR-amplified using universal primers. The amplified product was cloned and sequenced. The results revealed 20 of the sugarcane rhizosphere isolates as *P. plecoglossicida*, 2 as *P. fluorescens*, 2 as *P. libaniensis*, and 1 as *P. aeruginosa*. Thus, the 16S rDNA sequence analysis indicated that it could be used for the identification of fluorescent *Pseudomonas* and differentiation from other closely related species.

Phylogenetic Affiliation of Sugarcane Rhizosphere Isolates

A phylogram of the fluorescent *Pseudomonas* isolates was constructed based on the UPGMA with 1,000 bootstrap samplings. The analysis was performed with the sequences of the 25 isolates and 24 *Pseudomonas* reference isolates at

Table 2. Phenotypic characteristics of pseudomonad strains isolated from sugarcane rhizosphere.

Strain no.	Gram reaction	Shape	Motility	Sporulation	Pigment production	Oxidase test	Catalase test	Indole test	Citrate utilization	Arginine decarboxylase	Alanine decarboxylase	Gelatin liquefication	Growth		Glucose fermentation	Lactose fermentation	Ampicillin	Tetracycline	Chloramphenicol
													4°C	42°C					
S1	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	-	-	+	+	+
S2	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+
S3	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-
S4	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	-	-	+	-
S5	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	-	+	-	-
S6	-	Rod	Motile	-	+	+	+	-	+	+	+	-	-	-	+	+	-	+	+
S7	-	Rod	Motile	-	+	+	+	-	+	+	+	-	-	-	+	-	+	+	+
S8	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	-	-	+	+	-
S9	-	Rod	Motile	-	+	+	+	-	+	+	+	-	-	-	-	-	+	-	+
S10	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	+	-	-	+
S11	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	-	+	-	-	-
S12	-	Rod	Motile	-	+	+	+	-	+	+	-	+	-	+	+	-	-	-	+
S13	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+
S14	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	-	+	+	-	-
S15	-	Rod	Motile	-	+	+	+	-	+	+	-	+	-	+	-	-	+	+	-
S16	-	Rod	Motile	-	+	+	+	-	+	+	+	-	-	-	+	+	-	+	+
S17	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+
S18	-	Rod	Motile	-	+	+	+	-	+	+	-	+	-	+	+	-	-	-	-
S19	-	Rod	Motile	-	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+
S20	-	Rod	Motile	-	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+
S21	-	Rod	Motile	-	+	+	+	-	-	+	-	+	-	-	-	+	-	+	+
S22	-	Rod	Motile	-	+	+	+	-	+	+	+	-	-	+	+	-	+	-	+
S23	-	Rod	Motile	-	+	+	+	-	+	-	+	-	+	-	+	-	-	+	-
S24	-	Rod	Motile	-	+	+	+	-	+	+	-	-	+	+	+	+	+	-	+
S25	-	Rod	Motile	-	+	+	+	-	+	-	+	-	-	-	+	-	-	+	-

the selected region. The phylogenetic analysis of these isolates resulted in four distinct clusters (Fig. 1). Among them, cluster II formed a major group (20 out of 25) comprising *P. plecoglossicida* isolates. However, the type strains *P. plecoglossicida* AB009457 and *P. putida* D84020 were grouped in cluster II. On the contrary, a few other reference bacteria such as *P. cichorii* AB021398, *P. monteilii* AB021409, *P. oryzihabitans* D84004, and *P. fulva* D84015 were also represented in cluster II. The second major group was formed by cluster III comprising 10 isolates of which 8 were reference bacteria and 2 were isolates of *P. plecoglossicida* (S23) and *P. aeruginosa* (S25). Similarly, two isolates of *P. fluorescens* (S2 and S16) were grouped in cluster I and two isolates of *P. libaniensis* (S1 and S17) were grouped in cluster IV. Surprisingly, isolates S2 and S16 belonging to *P. fluorescens* did not share similarity with the *P. fluorescens* reference strain (D84013) but showed similarity with other reference isolates and were grouped in cluster I. These results suggest that genetic variation appears within the species.

16S rRNA Based Fingerprinting of PGPR *Pseudomonads*

The internal transcribed spacer (ITS) sequence in the rRNA operon of all of the selected PGPR isolates was amplified with ITS primers. PCR of the 16S–23S ITS region of all of the isolates gave only a single amplicon with an approximate size of 560 bp, confirming them as genus *Pseudomonas*. Furthermore, the 16S rDNA region was also amplified using genus-specific primers, resulting in the expected 960 bp amplicon. ARDRA analysis of the isolates resulted in different fingerprint patterns based on the different restriction enzymes. Using *Xma*I, *Alu*I, or *Nla*IV, 3 to 5 fragments were recorded for all of the isolates. However, all 25 isolates produced a monomorphic

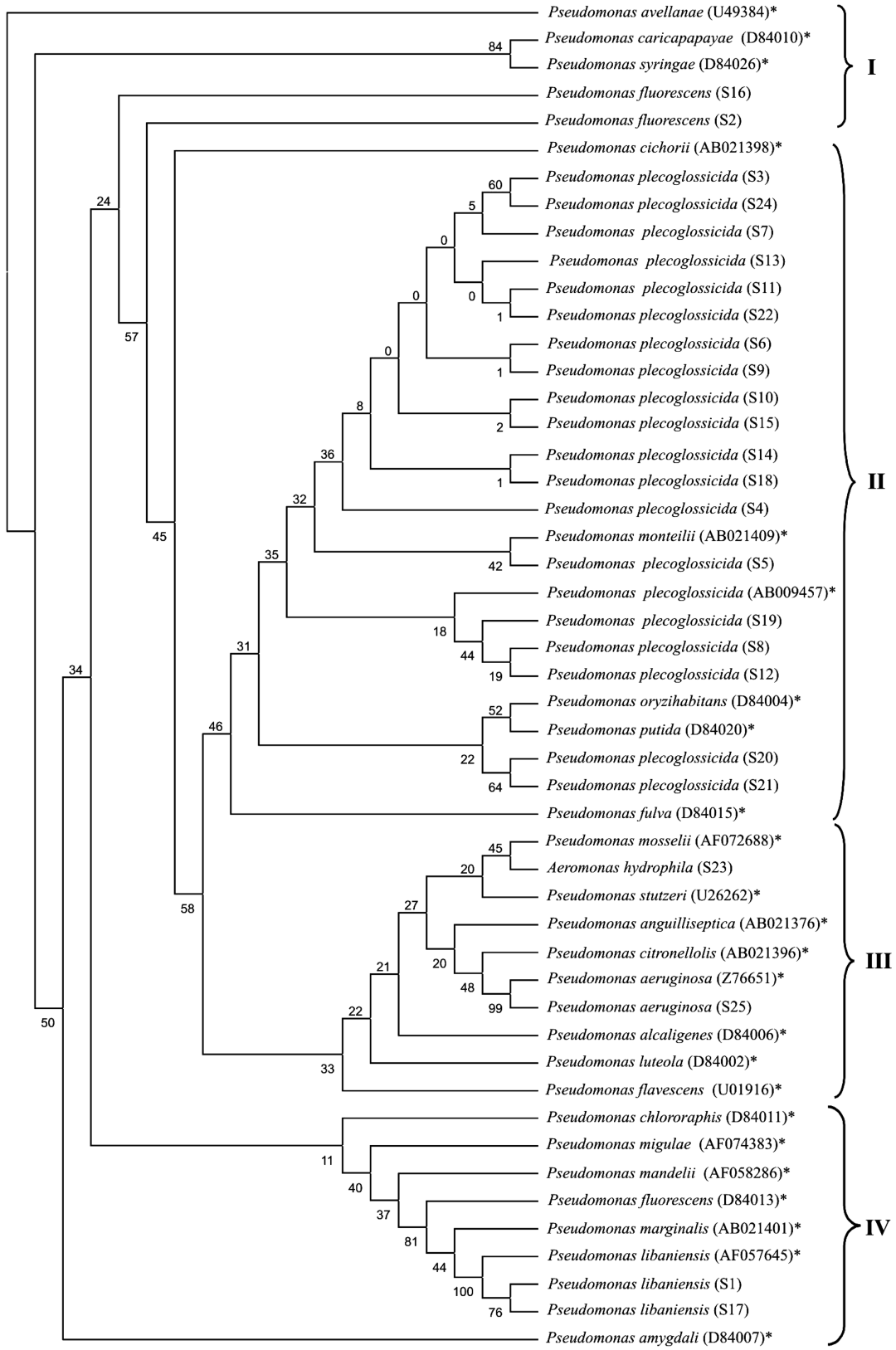

Fig. 1. Phylogenetic tree of fluorescent pseudomonad strains based on 16S rDNA sequences.
The tree was constructed using the neighbourjoining method. To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method.

Table 3. Genetic diversity of fluorescent *Pseudomonas* isolated from sugarcane rhizosphere.

Strain	16S rDNA-based identification	Accession No.[a]	Analysis of genetic diversity using different DNA fingerprint analyses				
			RAPD	ARDRA	ERIC	REP	BOX
S1	*P. libaniensis*	DQ095890	IV	III	IV	II	IV
S2	*P. fluorescens*	DQ095890	II	II	III	IV	III
S3	*P. plecoglossicida*	DQ095891	I	I	III	1V	II
S4	*P. plecoglossicida*	DQ095892	III	I	III	IV	I
S5	*P. plecoglossicida*	DQ095893	II	I	III	IV	III
S6	*P. plecoglossicida*	DQ095894	I	I	II	I	III
S7	*P. plecoglossicida*	DQ095895	I	I	I	III	IV
S8	*P. plecoglossicida*	DQ095896	III	I	I	I	IV
S9	*P. plecoglossicida*	DQ095897	II	I	IV	III	IV
S10	*P. plecoglossicida*	DQ095898	II	I	III	IV	IV
S11	*P. plecoglossicida*	DQ095915	IV	I	IV	III	IV
S12	*P. plecoglossicida*	DQ095899	III	I	IV	II	IV
S13	*P. plecoglossicida*	DQ095900	IV	I	IV	II	II
S14	*P. plecoglossicida*	DQ095901	I	I	II	I	IV
S15	*P. plecoglossicida*	DQ095902	I	I	I	IV	II
S16	*P. fluorescens*	DQ095903	I	II	IV	I	IV
S17	*P. libaniensis*	DQ095904	I	III	I	II	IV
S18	*P. plecoglossicida*	DQ095905	IV	I	III	IV	IV
S19	*P. plecoglossicida*	DQ095906	I	I	II	IV	II
S20	*P. plecoglossicida*	DQ095907	III	I	II	IV	II
S21	*P. plecoglossicida*	DQ095909	IV	I	I	IV	I
S22	*P. plecoglossicida*	DQ095908	I	I	II	I	III
S23	*P. plecoglossicida*	DQ095910	I	I	II	III	III
S24	*P. plecoglossicida*	DQ095882	I	I	I	II	III
S25	*P. aeruginosa*	DQ095912	IV	IV	I	II	IV

[a]Note: The sequences were deposited in the NCBI GenBank database.

ARDRA pattern when digested with *Xma*I. The *Alu*I-generated fingerprint also had a similar pattern, resulting in several isolates being found to be identical. However, the restriction enzymes *Nla*IV and *Pli*I produced an average of four bands that were less informative. In contrast, polymorphic banding patterns were observed with *Hae*III-digested products. Analysis of *Hae*III-generated patterns resulted in the recognition of four different ARDRA patterns, which grouped isolates belonging to the same species together: 20 isolates of *P. plecoglossicida*, 2 isolates of *P. fluorescens,* 2 isolates of *P. libaniensis*, and 1 isolate of *P. aeruginosa*. Therefore, the fingerprints generated by the *Hae*III ARDRA could be used to differentiate isolates belonging to different groups but failed to differentiate those belonging to the same species (Table 3).

Genomic Fingerprinting of PGPR *Pseudomonads*

Therefore, attempts were made to differentiate the isolates using whole genome fingerprinting techniques, beginning with RAPD. Initially, five different primers were used in the RAPD assay. Among them, three primers (pgs2, pgs3, and pgs4) were selected for further analysis because they gave readily interpretable and reproducible results from PCR amplification of genomic DNA. RAPD of the isolates revealed DNA bands ranging from 100 to 2,500 bp. Variations were clearly observed in the PCR products. The pgs2-amplified products produced clear and distinct polymorphic bands in greater numbers in comparison with the other two RAPD primers. Hence, pgs2-amplified products were used to construct the dendrogram and to study the genetic relatedness of the PGPR strains. Dendrogram analysis of pgs2-amplified products could clearly differentiate the majority of the isolates. For instance, 11 out of 20 isolates belonging to *P. plecoglossicida* were grouped in cluster I, whereas the remaining strains were dispersed across other clusters and shared their homology with other isolates (Table 3). In general, strains showed wide genetic variation and shared homology with distinct groups. The pgs2 RAPD banding pattern could be employed to differentiate closely related organisms; however, it failed to give clear, precise differences among strains belonging to the same subgroup.

REP-PCR Analysis of PGPR *Pseudomonas*

REP-PCR analysis of *Pseudomonas* strains yielded one to six distinct bands ranging from approximately 0.1 to 5 kb. Differences among the strains was assessed based on the banding pattern of the PCR products. Rep-PCR analysis of the S-series isolates revealed wide genetic diversity. Interestingly, a 3 kb DNA fragment was observed in all of the isolates. Dendrogram analysis of these strains resulted in four different clusters (Table 3). Ten out of 25 isolates were grouped in cluster IV, which had a common rooting at 66% similarity index. Among them, nine isolates belonged to the *P. plecoglossicida* group and one strain, S2, belonged to *P. fluorescens*. Interestingly, cluster II comprised six isolates representing different groups; namely, S12, S13, and S24 belonging to *P. plecoglossicida*; S1 and S17 belonging to *P. libaniensis*, and one strain, S25, belonging to the *P. aeruginosa* group. Despite the variation among the isolates belonging to different groups, they shared considerable similarity with the isolates of *P. plecoglossicida.*

Clusters I and III formed minor groups comprising five and four isolates, respectively. Surprisingly, isolate S16, which belonged to the *P. fluorescens* group, did not share any homology with S1, S2, and S17, which belonged to the *P. fluorescens* and *P. libaniensis* groups. On the contrary, they shared considerable similarity with the isolates of *P. plecoglossicida* in cluster I. REP-PCR analysis of the isolates belonging to the *P. fluorescens, P. libaniensis*, and *P. aeruginosa* groups showed variation and could be used to differentiate them. On the other hand, *P. plecoglossicida* isolates were distributed in all of the clusters, suggesting that this DNA fingerprinting profile was not very useful in discriminating differences among the isolates belonging to this group.

ERIC-PCR Analysis of PGPR *Pseudomonads*

ERIC-PCR products of all of the isolates generated a polymorphic banding pattern in comparison with REP and BOX-PCR products. The number of ERIC-PCR bands ranged from 2 to 10 with a unique banding profile, suggesting that this analysis was useful for differentiating the isolates. Dendrogram analysis of ERIC-PCR products resulted in four clusters (Table 3). Cluster I comprised seven isolates, among which five belonged to the *P. plecoglossicida* group, one to *P. libaniensis* (S17), and one to *P. aeruginosa* (S25). Although S25 shared more homology with the *P. fluorescens* and *P. plecoglossicida* groups in cluster I, the remaining 18 isolates were grouped in clusters I, II, and III. Isolates S2 and S16 belonging to the *P. fluorescens* group were grouped in clusters III and IV, respectively. Similarly, a strain of *P. libaniensis*, S1, did not share any homology with S17, another strain belonging to this group, but was grouped in a distinct cluster. On the other hand, cluster II comprised six isolates belonging to the *P. plecoglossicida* group and could be differentiated only with very minor variation in their DNA fingerprinting profiles. Interestingly, *P. plecoglossicida* isolates that were grouped in the other clusters exhibited a different DNA fingerprinting profile in comparison with the strains that were grouped in cluster II. Surprisingly, S21, S20, S3, and S13 belonging to *P. plecoglossicida* showed very minor variation but were still grouped in distinct clusters. This result confirms the existence of genetic diversity among the isolates belonging to the same group.

BOX-PCR Analysis of PGPR *Pseudomonas*

BOX-PCR analysis of the isolates differentiated the different species belonging to PGPR *Pseudomonas*. However, it was not as discriminatory as the other methods used in this study. Dendrogram analysis of the DNA fingerprinting pattern resulted in four different clusters (Table 3). Cluster IV formed the major group, comprising 12 isolates, among which were S1 and S17, belonging to the *P. libaniensis* group, 1 strain belonging to *P. fluorescens* (S16), and 1 strain belonging to *P. aeruginosa* (S25). The remaining eight isolates belonged to the *P. plecoglossicida* group, but the number of bands generated by the BOX-PCR pattern for this particular group was about 2–3 and ranged from 700 to 1,500 bp. Therefore, it was very difficult to differentiate the isolates of the *P. plecoglossicida* group. The second major group was formed by cluster III comprising six isolates; interestingly, it included a strain belonging to *P. fluorescens* (S2), but still shared some similarity with isolates of the *P. plecoglossicida* group. On the other hand, clusters II and I comprised five and two isolates, respectively. Analysis of these results revealed that BOX-PCR fingerprints were not very useful in differentiating isolates belonging to the *P. plecoglossicida* group.

Combined BOX-, ERIC-, and REP-PCR Analyses

To improve the resolution of the analysis of genetic diversity, a dendrogram was constructed by combining the results from ERIC-, REP-, and BOX-PCR. The combined rep-PCR analysis resulted in the differentiation of the sugarcane rhizosphere isolates belonging to the same species in the same group. The analysis of S-series isolates resulted in no grouping among them; few isolates shared more than 70% similarity, but variation could be observed in their DNA fingerprints that was sufficient to differentiate the isolates belonging to the same species. The isolates belonging to *P. fluorescens*, *P. libaniensis*, and *P. aeruginosa* groups were more clearly differentiated; however, they shared some similarity with *P. plecoglossicida*. All 20 isolates belonging to the *P. plecoglossicida* group were differentiated more efficiently than by the other methods employed in this study (Table 3 and Fig. 2). Analysis of these results gave more clear and refined genomic variation among the isolates in this study. Combined analysis of the

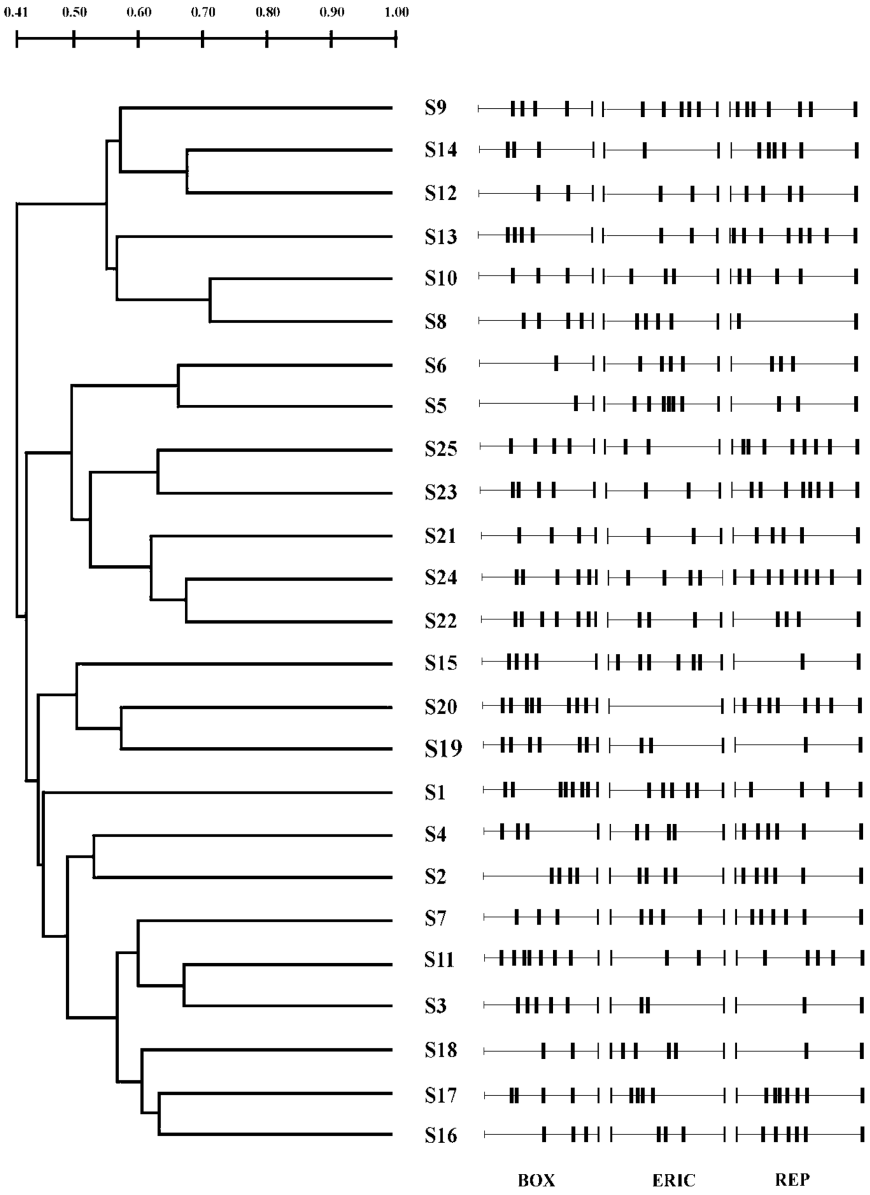

Fig. 2. Cluster analysis of combined ERIC-, REP-, and BOX-PCR genomic fingerprint patterns of functionally associated fluorescent *Pseudomonas* of sugarcane.
The UPGMA algorithm was applied to the similarity matrix generated with the Dice coefficient.

isolates confirmed the existence of genetic diversity among the PGPR fluorescent *Pseudomonas* isolated from the sugarcane rhizsophere. Thus, the dendrogram analyses of the combined results from all rep-PCR were useful in determining the genomic diversity of the isolates from the sugarcane rhizosphere.

DISCUSSION

Pseudomonas spp. are common soil bacteria easily cultured from most agricultural soils and rhizospheres. They have been intensively studied because of their ability to promote plant growth, either by directly stimulating the plant or by suppressing soil-borne pathogens. The predominance of fluorescent pseudomonads in the rhizosphere soils of plants has been reported, but the genetic variability of potent PGPR pseudomonads is just beginning to be explored [2, 39]. The role of fluorescent pseudomonads in agriculture has been a matter of interest because of their abundant population in the plant rhizosphere and their innate plant growth promotion properties.

The majority of PGPR organisms produce phytohormones and exhibit antifungal activity and phosphate solubilization

properties [8, 19]. The role of siderophores in the control of diseases has been well documented, and IAA production is an important criterion for determining the promotion of plant growth and development [26]. All of the isolates evaluated in this study produced IAA at varying concentrations ranging from 30 to 640 μg/100 ml. In addition, they also exhibited antifungal activity, phosphate solubilization, and denitrification properties. A significant number of isolates (23 out of 25) exhibited antifungal activity against phytopathogenic fungi, 18 out of 25 isolates solubilized insoluble phosphates, and 15 out of 25 isolates exhibited denitrification. Functional characterization of sugarcane rhizosphere-associated fluorescent pseudomonads suggested considerable variation in plant growth promotion properties.

In order to investigate the variation among the PGPR pseudomonads, a dichotomous key was employed to identify the isolates. The majority of the isolates were identified as *P. fluorescens*. Classification based on biochemical methods is not precise enough to distinguish similar organisms or to determine the phylogenetic relationships among them. In some cases, the identification provided by FAME profiling also did not correlate with established affiliations. Similarly, FAME analysis of the randomly selected isolates identified 9 out of 12 as *P. putida* biotypes and 3 as *P. syringae*. FAME analysis results did not agree with the biochemical characteristics for identification of the genera *Pseudomonas*, *Acinetobacter*, *Moraxella*, and *Alcaligenes* [23, 35]. This study also confirmed that phenotypic-based identification did not correlate with FAME results. These discrepancies are due to the inadequacies of the taxonomic libraries for closely related isolates rather than variability in the FAME profiles themselves. Phenotypic traits, such as carbon source utilization, cellular fatty acid profile, denitrification ability, and pigment formation, were not sufficient for the classification of the fluorescent pseudomonads up to the biovar level [39]. The genus containing fluorescent pseudomonads has several species, and there are limitations associated with the identification of species. Hence, phenotypic characterization could be reliable only for the confirmation of the genus *Pseudomonas* but not for the identification of species.

Alternatively, sequence analysis of ribosomal operons is a method of choice to determine the phylogenetic relationship among organisms. The 16S rRNA region was amplified using *Pseudomonas*-specific 16S rDNA primers and resulted in the expected 960 bp amplicon, confirming that all of the isolates belonged to this genus. Similarly, these primers were used to confirm the identity of 256 *Pseudomonas* isolates isolated from the rice rhizosphere with a salinity gradient [31]. 16S rRNA sequence analysis has been used to distinguish the species of the genus *Pseudomonas* and to delineate the *P. fluorescens* lineage including the species *P. fluorescens*, *P. aureofaciens*, *P. chlororaphis*, *P. marginalis*, *P. tolaasii*, and *P. viridiflava*. Similarly, 16S rRNA sequence analysis delineated 25 isolates as *P. plecoglossicida*, *P. fluorescens*, *P. libaniensis*, and *P. aeruginosa*. Phylogenetic analysis grouped all of the *P. plecoglossicida* isolates in cluster II and indicated shared homology with the reference isolates *P. plecoglossicida* AB09457 and *P. putida* D84020. On the contrary, isolates of *P. fluorescens* from the sugarcane rhizosphere, namely, S2 and S16, did not share 16S rRNA sequence similarity with the reference strain *P. fluorescens* D84013. These results clearly suggest the prevalence of genetic variation within the species of the isolates obtained from the sugarcane rhizosphere.

In earlier reports, *P. fluorescens* and *P. putida* have been identified as dominant species in rice and wheat rhizospheres [38, 39]. Similarly, Rangarajan *et al*. [29] identified the dominant population of fluorescent pseudomonads in the rice rhizosphere as *P. fluorescens*, *P. aeruginosa*, and *P. putida*. More recently, Naik *et al*. [18] identified *P. monteilii* as the dominant species in the banana rhizosphere along with other species of fluorescent pseudomonads. Interestingly, this study identified *P. plecoglossicida* as the dominant population in the sugarcane rhizosphere, along with *P. fluorescens*, *P. libaniensis*, and *P. aeruginosa*, as designated by 16S rRNA gene phylogeny. The dominant *P. plecoglossicida* reported in this study are believed to be the subgroup of *P. putida* [21].

To ascertain further the genetic variation among the isolates of PGPR fluorescent pseudomonads, different DNA fingerprinting analyses including ARDRA, RAPD, and rep-PCR were performed. The *Hae*III-generated ARDRA pattern grouped the *P. fluorescens*, *P. libaniensis*, *P. aeruginosa*, and *P. plecoglossicida* isolates separately into four distinct groups. However, differentiating among the isolates belonging to the same subgroup could not be achieved. On the other hand, RAPD analysis has given a better evaluation of the genetic diversity of PGPR fluorescent pseudomonads isolated from the same and different ecological niches. Picard *et al*. [25] analyzed 150 isolates of *Pseudomonas* spp. and identified 31 RAPD markers. The genotyping of *P. fluorescens* isolates producing antifungal compounds were carried out using RAPD [30]. Similarly, the primer pgs2 gave promising and useful results by generating many bands to differentiate almost all of the isolates of *P. fluorescens*, *P. libaniensis*, *P. aeruginosa*, and *P. plecoglossicida*. However, this analysis grouped all of the species belonging to the same group. Differentiation of isolates of the same species could not be achieved by this method.

Different types of repetitive sequence analysis were useful for evaluating the genetic diversity of the isolates from the sugarcane rhizosphere. Although the separate analyses of the rep sequences have their own level of genetic resolution to differentiate the organisms, they were not sufficient to resolve the fine genetic variation of all the

isolates. The results revealed a considerable degree of relative similarity between the isolates and varied depending upon the molecular method used. Because of the high degree of genetic diversity of the isolates, a lack of correlation between genotype and phenotypic (functional) traits has been observed in this study as reported in plant pathogenic fungi [17]. However, a combined analysis of REP-, ERIC-, and BOX-PCR fingerprints showed a higher cophenetic correlation coefficient than the independent dendrogram; thus, clustering was more accurate and consistent after combining the results. REP-, ERIC-, and BOX-PCR results can be combined as recommended by Rademaker *et al*. [27] to obtain more consistent clustering. In this manner, *P. syringae* pv. *persicae* could be distinguished in isolates from wild cherry using ERIC- and BOX-PCR, but not REP-PCR [32]. Similarly, combined analysis of our results revealed the largest number of distinct groups, which was useful for differentiating the genomic diversity of the isolates from the sugarcane rhizosphere. This method gave more clear and reliable results than the other methods employed in this study. The genotypic data presented here could aid substantially in the identification and selection of new PGPR pseudomonads. With rep-PCR, novel genotypes can be easily recognized and selected for more intensive analyses. Additionally, our screening program can be focused on genotypes that are known to be effective on sugarcane. This study is the first to report the high degree of interspecies diversity of *P. plecoglossicida* in the sugarcane rhizosphere and also indicates the heterogeneity of the fluorescent pseudomonads. Knowledge of the diversity of plant growth-promoting and biocontrol rhizobacteria is required not only for understanding their ecological role in the rhizosphere, but also for their utilization in sustainable agriculture. The data presented in this investigation revealed a high degree of functional and genotypic diversity among fluorescent pseudomonads in the sugarcane rhizosphere. These fluorescent pseudomonads with innate biofertilizing and biocontrol potential may play a vital role in plant growth promotion, disease control, and subsequent enhancement of crop yield.

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