Fluorescent pseudomonads were isolated from the rhizosphere of rice and sugarcane and examined for their biodiversity. All fifty strains of the fluorescent pseudomonads produced indole acetic acid. Among these pseudomonads, half of sugarcane rhizosphere isolates and one isolate from the rice rhizosphere exhibited phosphate solubilization activity. On the contrary, majority of the rice rhizosphere pseudomonads, and one isolate from sugarcane rhizosphere exhibited antifungal activity. These fluorescent pseudomonads were further classified based on their growth and biochemical characteristics. Those isolates that had same biochemical characteristics were distinguished by random amplification of polymorphic DNA (RAPD). These biochemical and molecular biological methods clearly differentiated fluorescent Pseudomonads of rice and sugarcane rhizosphere.

**Keywords:** Finger printing, Fluorescent pseudomonads, PCR, PGPR, Pseudomonas, RAPD, Rhizobacteria

The rhizosphere is the soil region where interactions between microorganisms and plant root occurs leading to beneficial, harmful or neutral effect on plants. Beneficial free-living or associative bacteria can be found among this microbial population. Bacteria that improve plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). These PGPR play an important role in increasing plant growth and crop productivity through fixing atmospheric nitrogen, synthesizing low molecular weight siderophores, secreting phytohormones (notably indole acetic acid; IAA), and solubilizing phosphates in the soil. They may also synthesize enzymes that modulate plant growth and development by influencing the level of plant growth hormones. Moreover, a particular PGPR may influence plant growth and development by using anyone or more of these mechanisms.

Fluorescent Pseudomonads are ubiquitous major group of PGPR that includes different species mainly *P. fluorescens, P. aeruginosa* and *P. putida*. Effect of *P. fluorescens* on a variety of crop plants has been reported. Several factors which influence the performance of an organism in the soil include production of 2,4-diacetylephloroglucinol (DAPG) which play a key role in the agricultural environments and their potential use in the sustainable agriculture. However, introduction of this organism in the field often fails because they are not able to colonize the roots or do not produce antibiotic compounds in the new environment. Thus, it is essential to understand the bacterial community structure in relation to environmental factors and ecosystem functions. This study reports the genetic diversity of the plant growth promoting fluorescent pseudomonads isolated from the rhizosphere of rice and sugarcane. The results clearly indicate that the strains isolated from different ecological niches generally showed wide genetic diversity despite some strains having similarity in their biochemical characteristics.

**Materials and Methods**

Isolation of bacterial strains — Fifty fluorescent pseudomonads were isolated from the rhizosphere of rice and sugarcane cultivated in two different locations (Sholavandan and Vakaikulam) around Madurai, India. The rhizospheric soil samples were homogenized in phosphate buffered saline (PSB) solutions and serially diluted and plated on King’s B medium (Pseudomonas isolation agar medium, Hi-Media, Mumbai). The plates were incubated at 30°C for 24 hr and colonies that fluoresced under UV light (λ= 356 nm) were selected and further purified on the same medium.
Analysis of biochemical and plant growth promoting characters — Biochemical tests for the cultures were carried out according to Collins et al.\textsuperscript{10}. Indole acetic acid production by the cultures was determined by the method of Patten and Glick\textsuperscript{11}. Phosphate solubilizing activity of the culture was determined after the growth of the culture on Pikovskaya's agar (Hi-Media, Mumbai) plates at 30°C for 72 hr. The colonies that exhibited halo zone around them were taken as positive for solubilization of tricalcium phosphate\textsuperscript{12}. For determining antifungal activity, agar plugs (4 mm diam) from actively growing fungal cultures namely, 	extit{Fusarium moniliformis} FM01 and 	extit{Rhizoctonia bataticola} RBA1 (obtained from Tamil Nadu, Agricultural University, Coimbatore, India) were taken and placed on the surface of the PDA plate. Simultaneously, cultures of fluorescent pseudomonads were streaked, (3 cm from the agar plug at sides towards the edge of petri plates). Plate inoculated with fungal agar plugs alone was used as control. The plates were incubated at 30°C until fungal mycelia completely covered the agar surface in control plate.

Identification of fluorescent pseudomonads— Fifty bacterial strains were grown in nutrient broth at 30°C for 16 hr and chromosomal DNA was extracted according to the method of Byun et al.\textsuperscript{13}. The 16S rDNA region was amplified using a specific forward primer [PA-GCATCCAAAACCTACTG; PF - TTGCTTCTCTTGGAG] and a reverse primer [CR - TACCTTGTTAAGACTTC] for identification of fluorescent pseudomonads. Further to confirm the identity of fluorescent pseudomonads, 16S-23S rRNA intervening sequence specific primers, such as ITS1-AAGTCGTACACAAGGTAG (forward) and ITS2-GACCATATATAACCCCAAG (reverse), were used. Furthermore, the 16S rDNA region was amplified using 	extit{Pseudomonas} genus specific 16S rRNA gene primers (20-mer forward 5'GGTCTGAGAGGA TGATCAGT and 18-mer reverse 5'TTAGGTCCACCTCGCGGCG)\textsuperscript{14}.

RAPD fingerprinting - Three different RAPD primers namely PGS2 [GGCTCGGTTC], PGS3 [GTAGGCCCGT] and PGS4 [AAGAGCGCG] were used for RAPD analysis. All primers used in this study were synthesized at Microsynth, Switzerland. PCR amplification in a 20 µl reaction mixture contained: 10X PCR buffer (with 2.5 mM MgCl\textsubscript{2}); 2 µl, 2 mM dNTP mixture 2 µl, 2 µM primer each 5 µl; Taq DNA polymerase 3U; H\textsubscript{2}O 8 µl and template DNA 50 ng. Thermal cycler (DNA Engine, MJ Research, USA) was used with the cycle parameters of 92°C for 45 sec, 28°C for 60 sec and 72°C for 30 sec. The total number of cycle was 40 with an initial denaturation step extended to 2 min and a final extension time of 10 min. PCR products were electrophoresed on agarose gel (2%), stained in ethidium bromide solution (0.5 µg/ml) for 30 min and photographed and analyzed using a Gel documentation system (Biorad, USA, Model 2000).

Similarity analysis — All PCR reactions were repeated for at least three times and the fingerprints were compared. Only RAPD bands, which appeared consistently, were evaluated. Calculation of pair wise coefficient similarity was based on the presence and absence of bands and cluster analysis with un-weighted pair group method arithmetic mean, UPGMA

Results

Analysis of biochemical and plant growth promoting properties — Fifty fluorescent pseudomonads were isolated from the rice (R) and sugarcane (S) rhizosphere soil samples of two different locations (Sholavadan and Vakaikulam, Madurai Dist., Tamil Nadu). They were classified based on their plant growth promoting characteristics namely phosphate solubilization, antifungal activity and IAA production. All fifty strains produced IAA but the level differed (Fig. 1). These strains showed significant difference in phosphate solubilization and antifungal activity. All except one (R13) of the rice rhizosphere isolates did not exhibit phosphate solubilization. On the contrary, 14 out of 25 sugarcane rhizosphere isolates exhibited phosphate solubilization. But, 17 of the rice rhizosphere isolates exhibited antifungal activity, while only one of the sugarcane rhizosphere isolates (S19) exhibited such activity. Among 35 strains that did not show phosphate-solubilizing activity, 17 of them exhibited antifungal activity.

Biochemical characterization of these fluorescent pseudomonads revealed that all strains possessed catalase, oxidase, and arginine hydrolysis activities. But they showed some variation in growth at different temperature, denitrification and indole tests. Therefore, they were further grouped based on their growth at 4°, 42°C and 30°C. Among the strains examined, 29 of them grew at 42°C, 8 of them at 4°C and 12 strains at 30°C and one at both 4° and 42°C.
Fig. 1—Flow chart showing classification of fluorescent pseudomonads based on the plant growth promotion properties and biochemical characteristics (Level of IAA produced by the strain: L—Low range <1.25 μg/ml; M—Moderate range from 1.3 to 1.5 μg/ml; H—High range above 1.5 μg/ml).
With respect to denitrification property, majority of them (45) were negative, while few (5) strains were positive. Therefore, combining both plant growth promoting properties and biochemical characters, all 50 strains were classified in nine groups (Group A-I); (Fig. 1). This approach sufficiently differentiated majority of the strains, but large number of strains in the group A (10 strains) and H (11 strains) did not show much variation in their biochemical and plant growth promoting property. Therefore, DNA polymorphism (RAPD) of the strains was examined to differentiate the strains within these groups (Fig. 2).

RAPD analysis - A dendrogram was constructed based on RAPD product of the strains in group A or H (Fig. 2) and relationship among the strains isolated from different plant rhizosphere was worked out. RAPD analysis of strains in the group H revealed that all eleven strains clustered in three subgroups (Fig. 3). Seven sugarcane rhizosphere strains (S42, S39, S31, S11, S40, S36, and S6) that exhibited similar biochemical properties, but found in two different subgroups. On the contrary, four different rice rhizosphere isolates (R41, R42, R15, and R44) were found in three different subgroups and shared some genetic homology with the sugarcane rhizosphere isolates.

RAPD analysis of Group A strains revealed that all of them in a single cluster, of which nine were sugarcane rhizosphere isolates and one was rice rhizosphere isolate (R13; Fig.4). This isolate (R13) showed considerable genetic homology with other sugarcane isolates and shared high degree of genetic homology with S34. These results suggested that bacteria isolated from entirely different rhizosphere could have considerable genetic relatedness.

Discussion

Microbial community in the plant rhizospheric soil is highly dynamic. Relative abundance of the microbial population in plant rhizosphere is shown to be dependent on the plant species soil types and environmental conditions. Several studies have reported that plant roots release variety of organic compounds namely sugars, amino acids, organic acids, fatty acids, enzymes, auxins and hydrogen cyanide. Recently, it has been shown that difference in root exudation at different stages of plant development influence the composition of microbial community in rhizosphere. Similarly in our study, the bacteria isolated from sugarcane and rice rhizosphere showed considerable difference in their phenotypic characteristics.

Although, all *Pseudomonas* strains were able to produce IAA, only some of them exhibited phosphate solubilization and antifungal activity. Significant
numbers of sugarcane rhizosphere isolates (14 out of 25) were able to solubilize the insoluble form of phosphates. However, large number isolates (24 out of 25) of rice rhizosphere did not have the phosphate solubilizing property. Similarly, all of the strains except one isolated from sugarcane rhizosphere did not exhibit antifungal activity, while 17 out of 25 isolates from rice rhizosphere exhibited antifungal activity. These results suggested that the population of Pseudomonas in rice rhizosphere and sugarcane rhizosphere differ in their phenotypic characteristics and hence the host plants influence the population of rhizosphere Pseudomonas. Plant rhizosphere is a dynamic environment in which many factors may affect structure and species composition of microbial communities that colonize the roots. Lemanceau et al.\textsuperscript{17} have also reported the effect of two plant species, flax and tomato on the diversity of soil population of fluorescent pseudomonads. The rhizosphere microbial communities vary in structure and species composition in different root location or in relation to soil type, nutritional status, age, stress, disease, and other environmental factors.\textsuperscript{18-21}

Based on the plant growth promoting properties and biochemical tests, all strains were classified into nine groups (A-I); (Fig.1). Twenty-nine of them could be differentiated based on the plant growth promoting properties and biochemical analysis, while remaining 21 strains showed least differences and placed in the group A and H. The biochemical characteristics are useful in distinguishing two different genera or species, whereas they are not efficient for studying the relationship and diversity of strains within the species. Legard et al.\textsuperscript{22} have also reported that the biochemical tests are not significant in distinguishing strain differences within the pathovars of P. syringae. Moreover, molecular analysis of genomic DNA of the organisms is useful for distinguishing the bacterial strains better at intraspecies level. Picard et al.\textsuperscript{23} have analyzed the biodiversity of 150 strains of Pseudomonas spp. and identified 31 RAPD markers. Since the differentiation by the biochemical methods was limited, Rameshkumar et al.\textsuperscript{24} have used PCR RAPD and RFLP analysis for the genotyping of P. fluorescens strains producing the antifungal compounds. Therefore, an attempt was made to differentiate the strains, and to study the genetic diversity of strains associated with sugarcane and rice in groups A and H based on RAPD analysis.

Analysis of the seven strains of sugarcane rhizosphere Pseudomonas in the group H showed wide genetic variation among them. However, few strains shared homology with the rice rhizosphere Pseudomonas. The strains of the rice rhizosphere (R) grouped in separate cluster still they share homology with the strains of sugarcane rhizosphere namely S40 and S36. Similarly, sugarcane isolate S11 shared high homology with the rice isolates and found in a separate group, suggesting the occurrence of similar bacterial strains from the different rhizosphere. In the group A, nine strains of sugarcane rhizosphere showed genetic diversity within them; however they shared homology with the strain R13 of the rice rhizosphere. Recently, several studies have reported the use of molecular fingerprints to analyze the dynamics in the rhizosphere during plant growth development and the effect of the plant species and the relative abundance of bacterial populations in the rhizosphere.\textsuperscript{25-26} Furthermore, a plant dependent bacterial diversity was demonstrated using molecular fingerprints.\textsuperscript{27,28}

The strains isolated from two-plant rhizosphere generally showed significant variation in their phenotypic and genotypic characters, with an exception of few identical strains. This would suggest the possible transfer of organisms from one rhizosphere to another and their establishment in different rhizosphere. Therefore, some identical strains may be present in different locations and they may share common fingerprints despite the diversity in their rhizosphere. Thus, RAPD analysis gives a
better understanding in evaluating the genetic diversity of the rhizosphere bacteria.

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