Diversity of fluorescent pseudomonads isolated from the forest soils of the Western Ghats of Uttara Kannada

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Attempts were made to assess the biochemical characteristics and genetic diversity of 52 fluorescent pseudomonads isolated from the soils of different forests in the Western Ghats of Uttara Kannada District, Karnataka. The isolates were tentatively identified up to species level based on biochemical and physiological characteristics. Among the isolates, 37 were tentatively identified as *Pseudomonas fluorescens*, 13 as *P. aeruginosa* and two as *P. aureofaciens*. Fifteen isolates were subjected to RAPD–PCR with eight random primers using UPGMA method. A high level of genetic diversity was observed in these isolates. The dendrogram constructed for the pooled data had six clusters and clearly grouped the biochemically identified species of pseudomonads together.

Keywords: Biochemical characteristics, fluorescent pseudomonads, forest soil, genetic diversity.

FLUORESCENT pseudomonads are ubiquitous bacteria that are common inhabitants of the rhizosphere and are the most studied group within the genus *Pseudomonas*. They can be visually distinguished from other species of *Pseudomonas* by their ability to produce water-soluble yellow–green pigments[1]. They comprise of *Pseudomonas aeruginosa*, the type species of the genus, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and the plant pathogenic species, *P. cichorii* and *P. syringae*[2].

All fluorescent pseudomonads fall into one of the five 'ribonucleic acid homology groups' as defined by rRNA–DNA complementation experiments. The G + C content[2] ranges from 58% to 68%. The plant beneficial pseudomonads are heterogenous in that they comprise of a collection of non-enteric, Gram-negative strains that are generally aerobic, non-fermenting and motile[1]. Fluorescent pseudomonads are well recognized as plant growth promoting rhizobacteria with nutrient mobilizing ability and as biocontrol agents against plant pathogens[3].

In the present communication, we report the biochemical characteristics and molecular diversity of fluorescent pseudomonads isolated from Western Ghats of Uttara Kannada District, Karnataka.

Fifty-two isolates of fluorescent pseudomonads isolated from the forest soils of Western Ghats, Uttar Kannada District and maintained as culture bank in the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad were used. Among the 52 isolates, ten each were from dry decidous forest (DDF), moist decidous forest (MDF), evergreen forest (EG) and degraded forest (DF); eight isolates were from the semi-evergreen forest (SEG) and four were from the coastal area (CF). All the isolates were subjected to biochemical and molecular characterization as detailed below.

All the 52 isolates were tested for their biochemical characters, viz. starch hydrolysis, lipid hydrolysis, casein hydrolysis, arginine hydrolysis, H_2S production, acid and gas production, indole production, catalase test, oxidase test, urease test, denitrification and gelatin liquefaction according to the procedures outlined by Cappuccino and Sherman[4]. The isolates were also examined for their ability to grow at 41°C and 4°C. They were identified tentatively up to species level based on these biochemical and physiological characteristics by referring to the *Bergey's Manual of Determinative Bacteriology*[1].

Further, 15 selected isolates [DDF 37, DDF 104(2), DDF 433(1), MDF 47(2), MDF 371(1), MDF 427, EG 200(1), EG 274(1), EG 374(1), SEG 76, SEG 177(1), SEG 386(1), DF 139(2), DF 440(1), DF 521(2)] and five reference strains [two each of *P. fluorescens* (NCIM 2099 and MTCC 103) and *P. aeruginosa* (MTCC 1934 and NCIM 2036) and one of *P. aureofaciens* (NCIM 2026); the reference strains with MTCC numbers were obtained from Indian Microbial Type Culture Collection, Chandigarh, whereas strains with NCIM numbers were obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune were subjected to diversity analysis using RAPD–PCR as detailed below.

The genomic DNA of each of the 20 fluorescent pseudomonads was isolated and purified following the methodology of Stahl and Flesher[5]. Purified genomic DNA extracts from the individual isolates were used as template DNA. Commercial kits of 'C', 'D' and 'O' random decamer DNA primers were obtained from Operon Technologies, Inc., USA. A total of eight random decamer DNA primers, viz. OPC-09 (CTCACCGTCC), OPD-02 (GGACCCAACC), OPD-03 (GTCGCCGTCA), OPO-06 (CCACGGGAAG), OPO-09 (TCCCACGCAA), OPO-13 (CTCAGAGTCC), OPO-15 (TGGCGTCCTT) and OPO-16 (TCGGCGGTTC) were used for RAPD analysis.

One primer at a time was used to study polymorphism between the 20 strains by RAPD–PCR assay with genomic DNA extracts from all strains as template DNA. Master mix was prepared afresh using 10× assay buffer with $MgCl_2$ (2.5 µl), dNTPs 2.5 mM (1.0 µl), primer (1.0 µl), *Taq* DNA polymerase 30 U/µl (0.33 µl) and sterile double-distilled water (19.17 µl) for all the strains to avoid handling errors. (The dNTPs and *Taq* DNA polymerase were obtained from M/s Bangalore Genie Pvt Ltd, Bangalore.) The master mix was distributed to 20 microfuge tubes of 0.5 ml capacity @ 24 µl per tube and 1 µl of template

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Table 1. Biochemical and physiological characteristics of fluorescent pseudomonads isolated from Western Ghats

Isolate	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
DDF 37	+++	–	–	–	+	+	–	–	+	+	+	+	–	–	+	–	*Pseudomonas fluorescens*
DDF 38	+++	–	–	–	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DDF 72	++	–	+	+	+	+	–	–	+	–	+	+	–	+	–	+	*P. aeruginosa*
DDF 97(1)	+++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
DDF 104(2)	++	–	+	+	+	–	–	–	+	–	+	+	–	+	–	+	*P. aeruginosa*
DDF 207(1)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DDF 246(2)	++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
DDF 252(1)	++	–	+	+	+	–	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
DDF 295(2)	+	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DDF 433(1)	+	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
MDF 47(2)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
MDF 68(2)	+++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
MDF 304(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
MDF 309(1)	++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
MDF 324(2)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
MDF 371(1)	++	–	+	+	+	+	–	–	+	–	+	+	–	+	–	+	*P. aeruginosa*
MDF 391(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
MDF 412(1)	++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
MDF 427	+	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
MDF 511(1)	++	–	+	+	+	–	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
EG 200(1)	+++	–	–	–	+	–	–	–	+	–	–	+	–	+	+	–	*P. aureofaciens*
EG 274(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
EG 282(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
EG 318(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
EG 332(1)	+++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
EG 335(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
EG 346(2)	++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
EG 374(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
EG 396(1)	++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
EG 432	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
SEG 61(1)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
SEG 76	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
SEG 134	++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
SEG 177(1)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
SEG 237(1)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
SEG 340(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
SEG 356(4)	+	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
SEG 386(1)	+	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DF 73	+++	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
DF 75	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DF 139(2)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DF 440(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DF 441(1)	++	–	–	–	+	–	–	–	+	–	–	+	–	+	+	–	*P. aureofaciens*
DF 505(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
DF 521(1)	+++	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
DF 521(2)	+++	–	+	+	+	–	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DF 521(3)	+++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
DF 521(4)	+	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*
CF 494(1)	+	–	+	+	+	+	–	–	+	+	+	+	–	+	–	+	*P. aeruginosa*
CF 494(2)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
CF 518(1)	++	–	+	+	+	+	–	–	+	+	+	+	–	–	+	–	*P. fluorescens*
CF 518(2)	+	–	+	+	+	+	–	–	+	–	+	+	–	–	+	–	*P. fluorescens*

A, Fluorescence; B, Starch hydrolysis; C, Lipid hydrolysis; D, Casein hydrolysis; E, Arginine hydrolysis; F, H_2S production; G, Acid production; H, Gas production; I, Indole production; J, Catalase test; K, Oxidase test; L, Gelatin liquefaction; M, Urease test; N, Denitrification; O, Growth at 4°C; P, Growth at 41°C and Q, Tentative identification. Fluorescence: +, Weak; ++, Medium; +++, High fluorescence. +, Positive, –, Negative.

DNA from the respective isolates was added to make the total reaction volume in each tube equal to 25 µl. PCR amplification was done following the method of Castrillo and Brooks[6] with slight modification [I stage: one cycle of denaturation at 94°C for 4 min; II stage: 35 cycles (consisting of denaturation at 94°C for 1 min; annealing at 37°C for 3 min and extension at 72°C for 2 min) and III stage: one cycle of extension at 72°C for 10 min]. After the completion of the required cycles of amplification, the contents were loaded onto 1.2% agarose gel for elec-

trophoretic analysis. The amplification profiles for all the primers were compared and bands of DNA fragments were scored as present (1) or absent (0), generating the 0, 1 matrices. Genetic similarity was estimated using Dice coefficient with NTSYS PC-2.0 software program[7,8]. Clustering was done and dendrograms were drawn following unweighted pair group arithmetic mean (UPGMA) routine, using the above program. Other parameters, viz. per cent polymorphism and the discrimination power of a primer were computed by the formulae given below:

$$\text{Per cent polymorphism} = \frac{\left[\begin{array}{c}\text{Total number of}\\ \text{polymorphic bands}\end{array}\right]}{\text{Total number of bands}} \times 100.$$

$$\begin{array}{l}\text{Discrimination}\\ \text{power of a primer}\end{array} = \frac{\left[\begin{array}{c}\text{Number of unique}\\ \text{RAPD phenotypes}\end{array}\right]}{\text{Total number of phenotypes}} \times 100.$$

The results of biochemical and physiological tests (Table 1) indicated that all the 52 isolates were positive for gelatin liquefaction, arginine hydrolysis and indole production; 48 isolates were positive for lipid and casein hydrolysis. While most of the isolates produced H_2S and were positive for oxidase and catalase tests, none was positive for starch hydrolysis, urease test, and acid and gas production. Fifteen isolates possessed denitrification ability and 13 showed growth at 41°C. Thirty-nine isolates showed growth at 4°C. All the 52 isolates showed fluorescence on King's B agar, but the intensity of fluorescence varied among the isolates. Based on these morphological, biochemical and physiological characters, 37 isolates were tentatively identified as *P. fluorescens*, 13 as *P. aeruginosa* and two isolates as *P. aureofaciens*.

Polymerase chain reaction (PCR) refers to the *in vitro* amplification of particular DNA sequences using arbitrary or specific primers and a thermostable DNA polymerase enzyme[9]. Randomly amplified polymorphic DNA (RAPD) involves the use of random primers in PCR reactions[10] and has been used increasingly to distinguish closely related organisms[11] based on polymorphism in the RAPD product patterns. The PCR-based RAPD markers help in diversity analysis as well as fingerprinting of an individual. Picard *et al.*[12] reported that RAPD–PCR analysis could be used as an effective tool to study the frequency and biodiversity of *Pseudomonas* strains.

The PCR amplicons of fluorescent pseudomonads (15 selected isolates and five reference strains) obtained from eight random primers produced 127 polymorphic bands across the 20 strains in the present study (Table 2). While the minimum number of bands (9) was produced by the primers OPD-02 and OPO-09, maximum number of bands (25) was produced by OPO-16 (Figure 1). All the primers showed 100% polymorphism. The differentiating power of the random primers on fluorescent pseudomonads was also analysed, which indicated that the primers OPC-09, OPO-06, OPO-13 and OPO-16 had the highest differentiating power of 100% (Table 3). These primers could be useful for differentiation of fluorescent pseudomonads in future studies.

The dendrogram constructed for the pooled data (Figure 2) clearly grouped the biochemically classified species of fluorescent pseudomonads. Rangarajan *et al.*[13] also analysed populations of *Pseudomonas* isolated from the rhizosphere soils of pearl millet, cotton and paddy grown in saline soils for their biochemical characters and genetic diversity using molecular tools, including RAPD and PCR–RFLP. They found majority of the isolates belonged to *P. pseudoalcaligenes* and *P. alcaligenes*, irrespective of the host rhizosphere.

The dendrogram constructed for the pooled data (Figure 2) indicated the existence of six clusters. The first cluster consisted of two reference strains of *P. fluorescens* (NCIM 2099 and MTCC 103) with a genetic similarity value of 0.18. The second cluster consisted of one of our isolate EG 200(1), which was tentatively identified as *P. aureofaciens* based on biochemical and physiological characters, and the reference strain of *P. aureofaciens* (NCIM 2026) with a genetic similarity value of 0.36. Similarly, the third cluster was formed by the isolate MDF 371(1), and the reference *P. aeruginosa* (NCIM 2036) at a genetic similarity of 0.13. The fourth cluster consisted of refer-

Table 2. Summary of RAPD analysis of selected fluorescent pseudomonads

Primer	Total number of bands	Total number of polymorphic bands	Per cent polymorphism
OPC-09	18	18	100
OPD-02	9	9	100
OPD-03	23	23	100
OPO-06	12	12	100
OPO-09	9	9	100
OPO-13	13	13	100
OPO-15	18	18	100
OPO-16	25	25	100
Total	127	127	

Table 3. Differentiating powers of primers used in RAPD analysis of fluorescent pseudomonad isolates

Primer	Total number of phenotypes	Total number of RAPD phenotypes	Differentiating power (%)
OPC-09	20	20	100
OPD-02	20	19	95
OPD-03	20	18	90
OPO-06	20	20	100
OPO-09	20	19	95
OPO-13	20	20	100
OPO-15	20	17	85
OPO-16	20	20	100

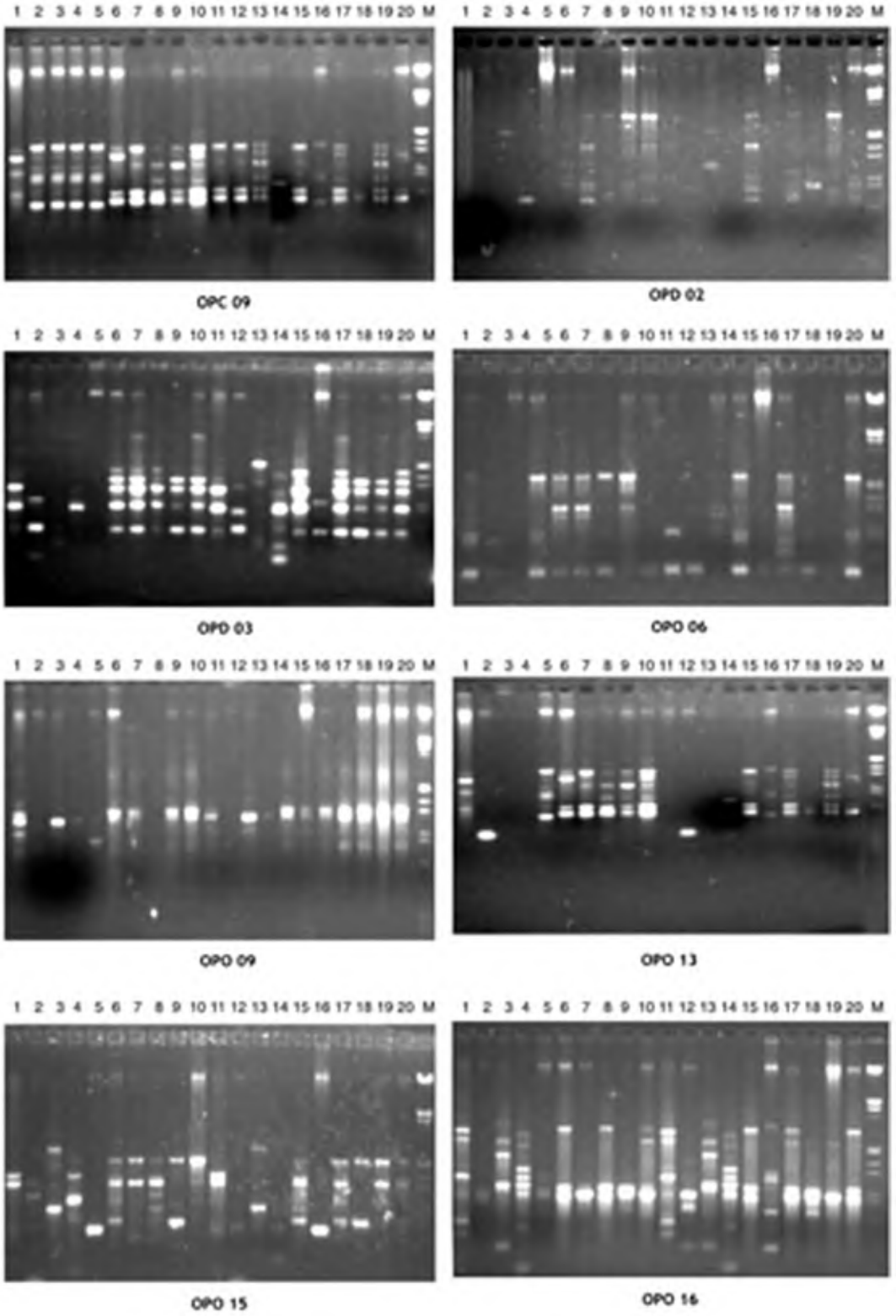

Figure 1. RAPD profiles of fluorescent pseudomonads with different primers. Lanes 1–20: NCIM 2099, NCIM 2036, NCIM 2066, MTCC 103, MTC 1934, DDF 37, MDF 47(2), SEG 76, DF 139(2), SEG 177(1), EG 274(1), DDF 104(2), EG 200(1), MDF 371(1), SEG 386(1), MDF 427, DDF 433(1), EG 374(1), DF 440(1), DF 521(2); M: λ *Hin*dIII digest.

ence strain *P. aeruginosa* (MTCC 1934) and the isolate DDF 104(2) at a genetic similarity of 0.30. The identity of these two native strains [MDF 371(1) and DDF 104(2)] in the third and fourth clusters as *P. aeruginosa*

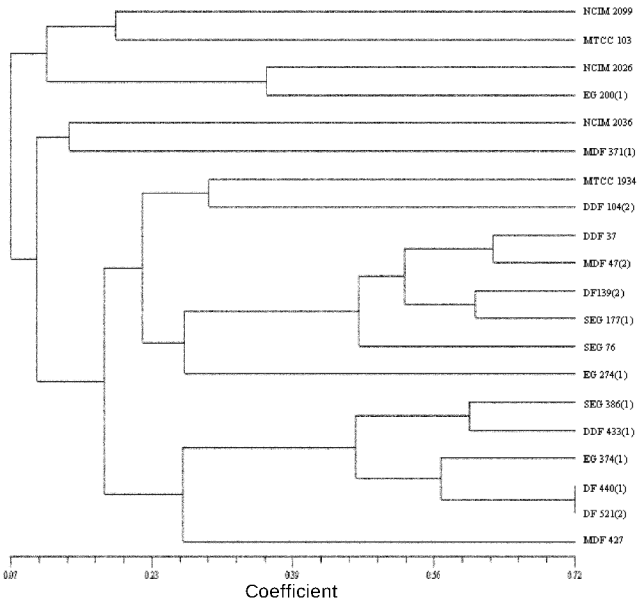

Figure 2. Dendrogram obtained for the pooled data.

based on their morphological, biochemical and physiological characters was also confirmed through their clustering with the reference strains of *P. aeruginosa*. Campbell *et al.*[14] used RAPD as a method for distinguishing *P. aeruginosa* strains and found high reproducibility and discriminating power with this technique. They were able to identify 131 distinct RAPD types among the 600 samples from patients with cystic fibrosis.

The fifth cluster formed by DDF 37, MDF 47(2), DF 139(2), SEG 177(1), SEG 76 and EG 274(1) at a genetic similarity of 0.26, consisted of four sub-clusters. DDF 37 and MDF 47(2) formed the first sub-cluster. The second sub-cluster was formed by DF 139(2) and SEG 177(1). The third and the fourth sub-clusters were formed by SEG 76 and EG 274(1) respectively.

The sixth cluster had three sub-clusters at a genetic similarity value of 0.26. Two isolates, viz. SEG 386(1) and DDF 433(1) formed the first sub-cluster, whereas EG 374(1), DF 440(1) and DF 521(2) formed the second sub-cluster. Only one isolate, MDF 427, formed the third sub-cluster. All the isolates that belonged to the fifth and sixth clusters were tentatively identified as *P. fluorescens*, which however diverged from the reference strains of *P. fluorescens* (NCIM 2099 and MTCC 103) at a genetic similarity coefficient of 0.26. The formation of different sub-clusters within the fifth and sixth main clusters by the *P. fluorescens* isolates indicates the existence of diversity among the isolates.

Using RAPD as a genetic tool, Suneesh[15] analysed molecular diversity of ten fluorescent pseudomonads from moist decidous forest soils of the Western Ghats and observed them to be distributed in five clusters. Though RAPD is used for molecular diversity analysis, according to Palleroni[16], popular methods of molecular analysis of natural microbial communities lack resolving power when it comes to the identification at the species level and they also fail to give sufficient information to infer the functions of newly isolated organisms. However, these methods have a role in surveying the composition of microbial communities and in the characterization of bacterial isolates. In the present study, we find a good match between the biochemical identification and grouping through RAPD–PCR analysis.

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