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RESEARCH ARTICLE

GENETIC DIVERSITY OF *PSEUDOMONAS* ISOLATES FROM GRASSLAND AND FOREST SOILS OF NORTHERN HIMALAYAS BASED ON 16S RRNA AND GENUS SPECIFIC REGION

***Asmita Rajwar and Manvika Sahgal**

Department of Microbiology, College of Basic Sciences and Humanities G.B. Pant University of Agriculture and Technology, Pantnagar Uttarakhand, India

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ABSTRACT

The genus *Pseudomonas* (sensu stricto) represents a group of microorganisms directly involved in functions conferring plant health. Specific detection of *Pseudomonas* species in the environment may help to gain a more complete understanding of the ecological significance of these microorganisms. In this present study, 16S rDNA and *Pseudomonas* specific gene region was amplified and it was found out that *Pseudomonas* specific PCR primer set; which amplify the 990bp region; is more specific for *Pseudomonas* sensu stricto. In UPGMA dendrogram based on ARDRA of 16S rDNA region, twelve isolates clustered separately from standard strains representing *Pseudomonas fluorescens* biovar 2, 3, 4 and *Pseudomonas fluorescens* CHA0 whereas remaining five were intertwined with reference strains used. Out of 17 isolates (PS1, PS2, PS3, PS4, PS5, PS6, PS7, PS8, PS9, PS10, PS11, PS12, PS13, PS14, PS15, PS16 and PS17) *Pseudomonas* specific region was amplified in 11 isolates (PS1, PS2, PS3, PS4, PS7, PS8, PS9, PS10, PS14, PS15 and PS17). In UPGMA dendrogram based on polymorphism in *Pseudomonas* specific primer, eleven isolates were placed in two major clusters. Isolates in cluster I could represent different biovars of *P. fluorescens* or subspecies of *P. aeruginosa* whereas cluster II represent a novel species in the genus *Pseudomonas*.

INTRODUCTION

The genus *Pseudomonas* includes bacteria that are metabolically versatile and live under diverse environmental conditions. In recent years, *Pseudomonas* strains have been studied with increasing interest because of their importance in ecological, medical, food and environmental sectors.

Pseudomonas is widespread in soil and has ability to colonize the rhizosphere of majority of plants. The genus *Pseudomonas* now popularly known as Pseudomonad's comprises high percentage of total rhizospheric bacterial population.

Some species of Pseudomonas plant growth promoting and plant pathogen suppressing functions (O'Sullivan and O'Gara, 1992; Keel *et. al*., 1996), a few are pathogenic to plants (Chatterton *et al*., 2004; Heydari and Pessarakli 2010) while yet others are opportunistic pathogens of animals or humans (Palleroni, 1992; Budzikiewicz, 1994). Hence they are good candidates for being utilized as bio fertilizers and bio control agents. Further they can be exploited in environmental protection by being used as in bioremediation of natural or xenobiotic compounds.

The genus Pseudomonas was described by Migula in 1894 and subsequently revised by Palleroni (1984). The taxonomic description of genus Pseudomonas given by Palleroni (1984) has since changed drastically and the members are now classified into more than 10 genera. Several members classified under these 10 genera have know come to be known as "Pseudomonad's" which means Pseudomonas like.

In first edition of Bergy's manual of systematic bacteriology, a total of 94 Pseudomonas nomen species have been listed. Moreover, species within the group Pseudomonads has been subdivided on the basis of rRNA similarities into five distinct rRNA homology groups; RNA homology group I includes species like Pseudomonas aeruginosa, P. fluorescens, P. syringae, P. putida, P. chlororaphis, P. stutzeri, P. alcaligens, P. pseudoalcaligens, P. mendocina and are referred to as genus Pseudomonas sensu stricto (Palleroni, 2008). Species of Pseudomonas representing RNA homology group II to V have been reclassified to genera Brevundimonas, Sphingomonas, Comamonas, Acidovorax, Hydrogenophaga, Telluria, Xanthomonas and Stenotrophomonas. The members of genus Pseudomonas sensu stricto have remarkable adaptability to different environmental conditions and this has lead to increase in number of species assigned to the so called Pseudomonas sensu stricto.

From agricultural point of view it is profoundly important to understand Pseudomonas diversity in soil and plant rhizosphere. This has been made easier as a result of polyphasic taxonomic approach which integrates the use of genotypic and phenotypic data.

This provides information regarding taxonomic relationships within closely related bacterial species. Microbial diversity and dynamics within soil and rhizosphere is influenced by various factors including soil characteristics and environmental conditions. The type of plant species growing can greatly affect soil micro flora. Rhizospheric bacterial diversity is affected by

* Corresponding author**: Asmita Rajwar**
Department of Microbiology, College of Basic Sciences and Humanities G.B. Pant University of Agriculture and Technology, Pantnagar Uttarakhand, India

different plant species growing in an Ecosystem (Comp ant *et. al*., 2005).

Polymorphism in 16S rDNA region and housekeeping gene regions has been used for analyzing diversity amongst closely related bacterial genera and species. This kind of analysis further leads to characterization, identification and reveal phylogenetic relationships amongst them.

The 16S rDNA gene from Pseudomonas spp. contain 1492 nucleotide position, of which 148 are variable and 65 positions of these are within three hypervariable regions. Primers based on these regions have been used to study the diversity of Pseudomonad isolates from different parts of the world (Moore *et. al*., 1996). Remarkable degree of variability has been reported among strains of species that are phylogenetically closely related based on restriction fragment length polymorphism (Ginard *et. al*., 1997; Rainey *et. al*., 1994).

The objective of this study was to study the genetic heterogeneity among Pseudomonas isolates from forest and grassland soils 'bugiyals' of hilly regions of Uttarakhand and to ascertain their relationships.

MATERIALS AND METHODS

Isolation of Bacterial cultures and Genomic DNA: In all seventeen bacterial isolates were recovered from soil samples grasslands of Pithoragarh (Latitude-29°35′N and Longitude-80°15′N, Altitude-1615 masl) and forests of Almora (Latitude 29°37′N and Longitude 79°40′E, Altitude 1250 masl) central Himalayan region in Uttarakhand using King's B and Gould's medium (Gould *et. al*., 1984). Four reference strains belonging to different biovars of P. fluorescens were also incorporated in the study. The genomic DNA was extracted by a modified method of Bazzicalupo and Fani (1995).

PCR amplification of target genes of 16S rDNA and Pseudomonas specific region

Genomic DNA was amplified by using two different PCR-primer sets- one set was of universal primers (Anzai *et. al*., 2000) and other one was of Pseudomonas specific PCR (Ps-PCR) primers (Widmer *et. al*., 1998). The universal primer set included forward primer GM3f (5'AGAGTTTGATCMTGGC 3') and reverse primer GM4r (5' TACCTTGTTACGACTT 3') for the amplification of 1492 BP region of the 16S rDNA gene. A 50µl of reaction mixture included, 5 µl (5-10 ng) of bacterial DNA as template, 5 µl of 10X buffer for Taq DNA Polymerase (100 mM of TRIS-HCl and 15 mM $MgCl_2$), 1 µl of $MgCl_2$ (25 mM), so that the final concentration of Mg^{++} is 2 mM, 0.25µM of each primer, 400 µM of each dNTPs and one unit of Taq DNA polymerase (BANGALORE GENEI, INDIA). The reaction condition includes an initial denaturation of 7 min at 95^0C, followed by 35 cycles of 1 min at 94^0C, 1 min at 52^0C and 1 min at 72^0C with the final extension of 5 min at 72^0C.

Ps-PCR primer set included forward primer, Ps-for (5' GGTCTGAGAGGATGATCAGT 3') and the reverse primer, Ps-rev (5' TTAGCTCCACCTCGCGGC 3') for amplification of 990bp region of 16S rRNA gene. For getting amplification from Ps-PCR primer set, a 50µl of reaction mixture included, 5 µl (5-10 ng) of bacterial DNA as template, 5 µl of 10X buffer for Taq DNA Polymerase (100 mM of TRIS-HCl and 15 mM $MgCl_2$), 2.5 µM of each primer, 250 µM of dNTPs and one unit of Taq DNA polymerase (BANGALORE GENEI, INDIA). The reaction condition includes an initial denaturation of 5 min at 95^0C, followed by 35 cycles of 1 min at 94^0C, 1 min at 57^0C and 1 min at 72^0C with the final extension of 10 min at 72^0C. 45 µl of reaction mixture was added in each PCR tube containing 5 µl templates DNA. The amplification was carried out on Gen Amp PCR System 9700 (APPLIED BIOSYSTEMS). Amplified DNA was electrophoresed in 0.8 % agarose gel at 80 mA for 1h and visualized under UV gel documentation system Gel Doc Mega (BIOSYSTEMATICA).

Polymorphism in 16S rDNA and Pseudomonas specific region Restriction digestion of complete 16S rDNA region (1492bp) and Pseudomonas specific gene region (990bp) amplicon was set in 25 µl reaction mixture containing 20 µl aliquot of an amplicon of all the PCR reactions from both the primers as template, 1X enzyme buffer and 1U/rxn of each tetra cutting restriction enzymes Alu I, Rsa I and Taq I. PCR amplicons obtained using universal primers were digested with Alu I and Rsa I at 37^0C for 2h and Taq I at 65°C for 4h while amplicons obtained using Pseudomonas specific primers were digested with RsaI at 37^0C for 2h and Taq I at 65°C for 4h.

Enzymes were inactivated by mixing the loading dye and kept at -20°C. The products were analyzed by agarose gel (2.5%) electrophoresis carried out at 80V for 2/3 run of gel.

Phylogenetic relationships of the bacterial isolates

DNA bands generated by digestion of amplified 16S rDNA with the three restriction enzymes AluI, RsaI and TaqI and amplified Pseudomonas specific gene region with RsaI and TaqI were used to construct a UPGMA dendrogram using NTSYSpc version 2.0 software calculating Jaccard's similarity coefficient. Bands were scored for their presence (1) and absence (0), ignoring their intensities.

RESULTS

Polymorphism in amplified 16S rDNA region When 16S rDNA region of 17 bacterial isolates from grassland and forest soils (Table 1) and 4 standard strains representing P. fluorescens (Table 2) were amplified using universal 16S rDNA primers, a distinct band of 1492bp was observed (Fig.1). Out of the 17 isolates, ten isolates PS1-PS10 were from grassland soil, five isolates, PS11, PS12, PS14, PS15 and PS16 were from Pine forest soil and two isolates PS13 and PS17 were from Quercus forest soil.

On the basis of ARDRA profiling, all the seventeen strains were placed into five major clusters and each having two or three subclusters (Fig4).

Cluster I includes PS1 and PS3 bifurcated at a distance of 0.73 on Jaccard's scale. Cluster II also showed similar pattern with the isolates asPS4 and PS11 were placed at a distance of 0.70 on Jaccard's scale.

Cluster III was further furcated in two subclusters. PS5 and PS6 were 100% similar and were present in subcluster I. Isolates PS12 and PS13 were 100% related to each other and hold a place in subcluster II while present at a distance of 0.84 of Jaccard's scale to the subcluster I. Cluster IV involves two subclusters and PS2 as an outlying branch to the whole cluster. PS7 and PS8 were 100% similar and were present in subcluster I while *P. fluorescens* bv2 and *P. fluorescens* bv3 were 90% similar and present at a distance of 0.75 to isolates PS7 and PS8. Isolates PS10 and PS17 were present at a distance of 0.73

Table 1 Presumptive *Pseudomonas* isolates and their genotypic characterization

Isolates	Geographical location	Ecosystem	Rhizosphere	16S rDNA cluster	*Pseudomonas* specific cluster
PS1	Pithoragarh	Grassland	Soil	C I	C I
PS2	Pithoragarh	Grassland	Soil	C IV	C III
PS3	Pithoragarh	Grassland	Soil	C I	C II
PS4	Pithoragarh	Grassland	Soil	C II	C II
PS5	Pithoragarh	Grassland	Soil	C III, SC I	-
PS6	Pithoragarh	Grassland	Soil	C III, SC I	-
PS7	Pithoragarh	Grassland	Soil	C IV, SC I	C II
PS8	Pithoragarh	Grassland	Soil	C IV, SC I	C II
PS9	Pithoragarh	Grassland	Soil	C IV	C III
PS10	Pithoragarh	Grassland	Soil	C IV, SC III	C III
PS11	Almora	Pine	Soil	C II	-
PS12	Almora	Pine	Soil	C III, SC II	-
PS13	Almora	Quercus	Soil	C III, SC II	-
PS14	Almora	Pine	Soil	C V, SC I	C II
PS15	Almora	Pine	Soil	C III	C I
PS16	Almora	Pine	Soil	C IV	-
PS17	Almora	Quercus	Soil	C IV, SC III	C III

C = cluster SC = subcluster

Table 2 Reference strains and their source

Reference strains	Names used in this study
P.fluorescens bv 2	Standard 2
P.fluorescens bv 3	Standard 3
P.fluorescens bv 4	Standard 4
P.fluorescens CHAO	Standard CHAO

And 0.63 respectively to sub cluster I and were included in sub cluster II.

PS14 and standard *P. fluorescens* bv 4 were 33% related and were included in sub cluster I of cluster V but only 27% related to standard *P. fluorescens* CHA0 which was present as sub cluster II.

Polymorphism in Pseudomonas specific region of bacterial isolates

Out of seventeen isolates 990bp *Pseudomonas* specific region was amplified in only eleven isolates namely PS1, PS2, PS3, PS4, PS7, PS8, PS9, PS10, PS14, PS15 and PS17. This 990bp region was specific for RNA group I *Pseudomonas* (*sensu stricto*) hence it may be concluded from the result of amplification that PS5, PS6, PS11, PS12, PS13 and PS16 are not within *Pseudomonas* (*sensu stricto*) group.

The ARDRA profiling of these 11 isolates suggest that phylogenetic positions of these *Pseudomonas* isolates from different soils were interlinked with that of the four standards (fig5).

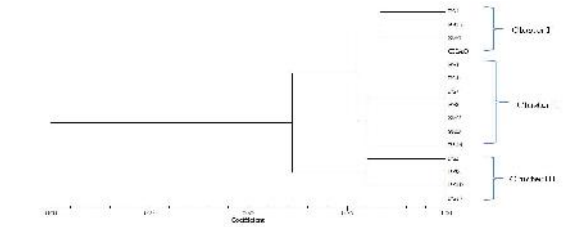
According to this dendrogram, all the 11 *Pseudomonas* isolates can be divided into six species or subspecies of *Pseudomonas.* Cluster I include PS1, PS15 and *P.* fluorescens bv 4 and P. fluorescens CHA0. PS15 was 100% similar to standards P. fluorescens bv 4 and P. fluorescens CHA0 which were 89% related to PS1.

Cluster II consist isolates PS3, PS4, PS7, PS8 and reference strains *P. fluorescens* bv 3 and which were 100% similar and 80% similar to PS14.

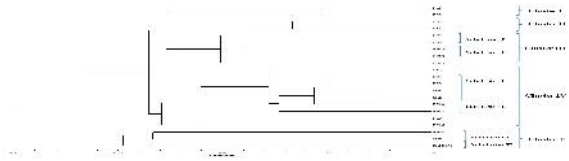
Cluster III involve isolates PS9, PS10 and PS17 which were 100% similar with each other and 80% similar to PS2.

Comparison of phylogenetic trees deduced from polymorphism in 16S rDNA and Pseudomonas specific gene region

The comparison of the phylogenetic trees obtained by ARDRA profiling of the two gene regions; 16S rDNA and *seudomonas* specific gene region was done.



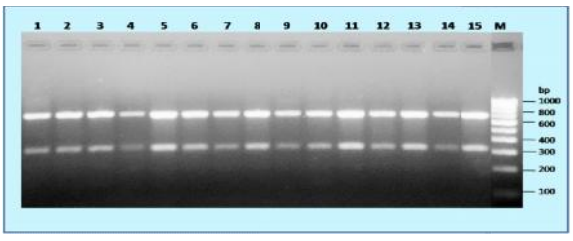
(a)



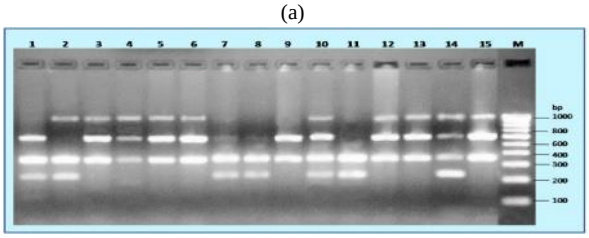
(b)

Fig 1(a)16S rDNA amplification using universal primers and **(b)** 16S rDNA Pseudomonas specific region amplification using Pseudomonas specific primers. Lane 1-17, PS1-PS17, Lane 18, P. fluorescens bv 2, Lane 19, P. fluorescens bv 3, Lane 20, P. fluorescens bv 4, Lane 21, P. fluorescens CHA0, Lane M, Marker (Lambda DNA/EcoRI/HindIII double digest).

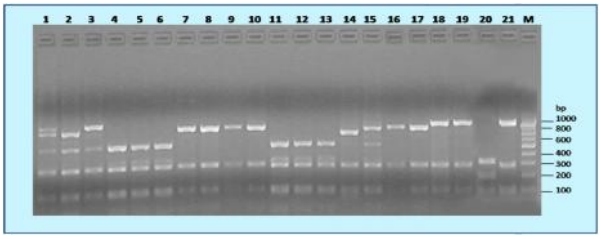
It was observed that the isolates get more defined and arranged in less number of clusters with the *Pseudomonas* specific gene region in comparison to ARDRA profile of 16S rDNA gene region. The depth of the branches also become less i.e. similarity percentage among the isolates was increased. A different result obtained from the phylogenetic trees of these two genes was that the standard stains of *P. fluorescens* used in the study were placed with different set of isolates in both the trees.



(a)



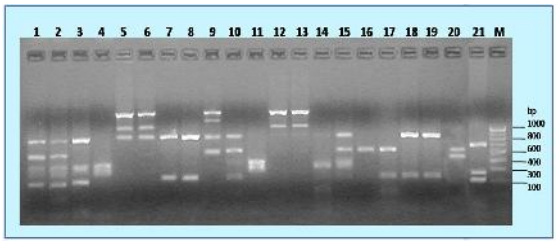
(b)



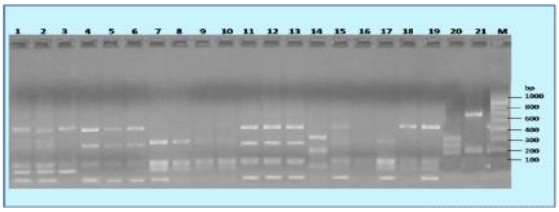
(c)

Fig 2ARDRA of presumptive Pseudomonas isolates (amplified with universal primers) from soil of Uttarakhand along with authentic strains of Pseudomonas. Lane 1-17, PS1-PS17, Lane18, P. fluorescens bv 2, xane 19, P. fluorescens bv 3, Lane 20, P. fluorescens bv 4, Lane 21, P. fluorescens CHA0, Lane M, Marker (100bp ladder) with restriction end nucleases (a) AluI (b) RsaI (c) TaqI.

In phylogenetic tree obtained by 16S rDNA region standard stains of *P. fluorescens* were placed with isolates PS7, PS8, PS9, PS10, PS14, PS16 and PS17 while these were placed with isolates PS1, PS3, PS4, PS7, PS8, PS14 and PS15 in the tree obtained with ARDRA profile of Pseudomonas specific gene region.



(a)



(b)

Fig 3 ARDRA of Pseudomonas isolates (amplified with Pseudomonas specific primers) from soil of Uttarakhand along with authentic strains of Pseudomonas. Lane 1-4, PS1-PS4, Lane 5-8, PS7-PS10, Lane 9-10, PS14-PS15, Lane 11, PS17, Lane 12, P. fluorescens bv 2, Lane 13, P. fluorescens bv 3, Lane 14, P. fluorescens bv 4, Lane 15, P. aeruginosa, Lane 16, P. fluorescens CHA0, Lane M, Marker (100bp ladder) with

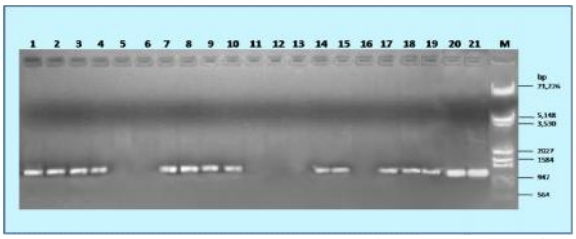


Fig 4 Combined UPGMA dendrogram of 16S rRNA gene of Pseudomonas isolates and reference strains on the basis of ARDRA with AluI, RsaI and TaqI

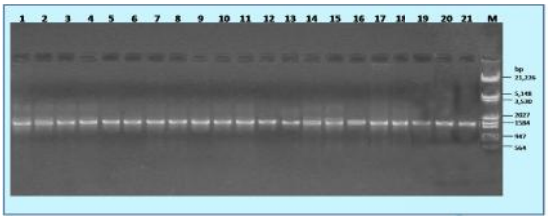


Fig 5 Combined UPGMA dendrogram of Pseudomonas specific region of Pseudomonas isolates and reference strains on the basis of ARDRA with RsaI and TaqI

DISCUSSION

In this study, genetic diversity among seventeen presumptive Pseudomonas isolates was assessed using ARDRA of 16S rDNA region and Pseudomonas specific region. The UPGMA dendrogram based on ARDRA of 16S rDNA region with three restriction enzymes *Alu* I, *Rsa* I and *Taq* I revealed significantly high diversity within the isolates as evident by the depth of branches in the dendrogram. It was observed that relationship of isolates from soils of grassland, Pine and Quercus forest were intertwined.

On the basis of 16S rDNA ARDRA analysis, reference strains Pseudomonas fluorescens bv 4 and Pseudomonas fluorescens CHAO form the separate cluster. This can be explained as the 16S rDNA region of all the isolates was restricted by three tetra cutter enzymes, each recognizing only 4bp site and each successive restriction is after 260bp i.e., the 16S rDNA region of 1500bp was restricted by one enzyme at approx. six sites. Thus polymorphism in only 24bp is accounted while drawing relationship. Hence, because of this reason Pseudomonas fluorescens bv 4 and Pseudomonas fluorescens CHAO were placed close together. This is exemplified in a study where a new Pseudomonas species, P. balearica was identified from the seven genomovars of P. stutzeri (Bennasar *et. al*., 1996). The evolutionary distances between strains belonging to the same genomovar were always less than 0.46 but the evolutionary distance between strains of P. stutzeri representing genomovar 3 AN10 and genomovar 6 LS401 was 4.44, hence genomovar 6 LS401 was described as a different species.

This 16S rDNA analysis revealed that these 17 bacterial isolates can be categorized in approximately 12 species of presumptive Pseudomonas isolates (Locatelli *et. al*., 2002). In a previous study when 16S rRNA sequence analysis of 128 valid and invalid Pseudomonas species was undertaken, fifty-seven species belonged to the genus Pseudomonas (sensu stricto) whereas remaining were related to other genera, which were placed in four subclasses of the Proteobacteria (α, β, γ, and γ-β subclasses) (Anzai *et. al*., 2000).

In a study conducted by Mulet and colleagues (2011), it was found out that when 16S rDNA analysis was conducted, the highest number of isolates was affiliated with the species P. stutzeri. This study was supported along with rpoD gene sequence analysis. The highly specific nature of the rpoD gene sequence even discriminated between genomovars (gv) within the P. stutzeri complex.

The sequence analysis of the 16S rRNA gene is widely employed for the identification of bacteria; however, this region is not satisfactorily discriminating between the species of *Pseudomonas*. Phylogenetic studies have highlighted that inferred phylogenies based on the 16S rRNA gene lack resolution at the intrageneric level because of its low rate of evolution. Hence, to conclude the study Pseudomonas specific gene region was amplified.

The ARDRA profile of Pseudomonas specific region showed that PS1 (isolated from grassland) and PS15 (isolated from Pine forest soil) were 89% similar though they are from two totally different geographic locations and were placed together with P. fluorescens bv 4 and P. fluorescens CHAO. Isolates PS2, PS9, PS10 and PS17 were 80% similar with each other and placed distantly from all the other isolates and reference strains. This indicates that they may be different species or pathovars or biovars of different species. Their further

characterization using 16S rDNA sequencing could ascertain their taxonomic position.

The Ps-PCR primer set amplified 990bp Pseudomonas specific region which is specific for Pseudomonas (sensu stricto) representing RNA homology group I. In our study, out of seventeen isolates, eleven isolates showed amplification by Pseudomonas specific primers (Widmer *et. al*., 1998). Pseudomonas has been extensively classified through PCR amplification of Pseudomonas specific 16S rDNA region. Amplification of 990bp Pseudomonas specific region in standard strains has confirmed this (Brosch *et. al*., 1996; Kersters *et. al*., 1996; Palleroni 1992).

Genetic diversity depicted in a UPGMA dendrogram based on ARDRA of 16S rDNA region is considerably reduced in a UPGMA dendrogram based on polymorphism in Pseudomonas specific region (Widmer *et. al*., 1998). The results concluded that approximately 12 species or subspecies which are observed by ARDRA of 16S rDNA are reduced to about six species, subspecies or biovars or pathovars by restriction analysis of Pseudomonas specific region. The similarity % among isolates increased. Thus Pseudomonas specific region is more applicable in taxonomic identification of Pseudomonas from agro forestry system. Although the Pseudomonas specific primer pair amplifies the region specific for Pseudomonas sensu stricto but when restriction digestion of Pseudomonas specific region of eleven positive isolates was performed, three major groups were formed. Thus these eleven isolates were not 100% similar and could represent new species or biovar of same species within Pseudomonas sensu stricto. Hence, the Pseudomonas specific-PCR protocol in conjunction with RFLP analysis, sequence determination and phylogenetic analyses may be used to compare Pseudomonas population structures from a variety of ecosystems and provide further insight about the occurrence, potential roles, and possible unidentified subgroups of this genus in different ecosystems (Mulet *et. al*., 2009, Mulet *et. al*., 2010). In addition, it may represent a rapid assay for confirmation of members of the genus Pseudomonas sensu stricto.

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