RESEARCH ARTICLE

Novel Phl-producing genotypes of finger millet rhizosphere associated pseudomonads and assessment of their functional and genetic diversity

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Abstract

Genetic diversity of *phlD* gene, an essential gene in the biosynthesis of 2,4-diacetylphloroglucinol, was studied by restriction fragment length polymorphism (RFLP) in 20 Phl-producing pseudomonads isolated from finger millet rhizosphere. RFLP analysis of *phlD* gene displayed three patterns with HaeIII and TaqI enzymes. *phlD* gene sequence closely correlated with RFLP results and revealed the existence of three new genotypes G, H and I. Further, the phylogenetic and concatenated sequence analysis of the 16S rRNA, *rpoB, gyrB*, *rpoD* genes supported the hypothesis that these genotypes G, H and I were different from reported genotypes A–F. In all phylogenetic studies, the genotype G formed a distant clade from the groups of *Pseudomonas putida* and *P. aeruginosa* (*sensu strictu*), but the groups H and I were closely related to *P. aeruginosa/P. stutzeri group*. The Phl-producing pseudomonads exhibited antagonistic activity against *Pyricularia grisea* (TN508), *Gaeumannomyces graminis* (DSM1463), *Fusarium oxysporum* (DSM62297), *Xanthomonas campestris* (DSM3586) and *Erwinia persicina* (HMGU155). In addition, these strains exhibited various plant growth-promoting traits. In conclusion, this study displays the existence of novel Phl-producing pseudomonads genotypes G, H and I from finger millet rhizosphere, which formed taxonomically outward phylogenetic lineage from the groups of *P. putida* and *P. aeruginosa* (*sensu strictu*).

Introduction

Pseudomonas sp. are dominant plant-associated bacteria in the group of Gammaproteobacteria, which are considered as promising plant growth-promoting rhizobacteria (PGPR). They are common inhabitants of rhizosphere, phyllosphere, can survive a broad range of environmental conditions and also highly influence the soil and plant health (Iavicoli *et al.*, 2003; Naik *et al.*, 2008; Prabavathy *et al.*, 2011).

Pseudomonads provide effective protection against bacterial and fungal pathogens, parasites and certain nematode infections (Chernin & Chet, 2002; Haas & Defago, 2005; Yin *et al.*, 2013). The biocontrol potential of pseudomonads is directly correlated with production of several antibiotics (Chin-A-Woeng *et al.*, 2003; Mazurier *et al.*, 2009; Upadhyay & Srivastava, 2011), among which 2,4-diacetylphloroglucinol (2,4-DAPG) has gained much attention as it is reported to inhibit a wide spectrum of bacterial and fungal plant pathogens (Immanuel *et al.*, 2012; Mishra & Arora, 2012; Almario *et al.*, 2013; Yin *et al.*, 2013). 2,4-DAPG is also reported to induce systemic resistance and systemic tolerance in the host plants (Iavicoli *et al.*, 2003). Phl-producing pseudomonads have been recovered worldwide from different rhizospheres (Keel *et al.*, 1996; McSpadden Gardener *et al.*, 2000; Picard *et al.*, 2000; De La Fuente *et al.*, 2006; Velusamy *et al.*, 2006; Immanuel *et al.*, 2012), and their contribution to the suppression of soil-borne plant pathogens has been reported (Sharifi-Tehrani *et al.*, 1998; Haas & Defago, 2005).

Synthesis of 2,4-DAPG is regulated by *phl* gene cluster, which consists of six open reading frames *phlACBDEFG* (Bangera & Thomashow, 1999). Among the biosynthetic loci, *phlD* gene is the crucial biosynthetic gene which codes for polyketide synthase, involved in the synthesis of

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monoacetylphloroglucinol (precursor for 2,4-DAPG synthesis) from acetoacetyl-CoA (Bangera & Thomashow, 1999), and is well conserved among all 2,4-DAPG producers worldwide (Picard & Bosco, 2003; De La Fuente *et al.*, 2006; Velusamy *et al.*, 2006; Frapolli *et al.*, 2007; Paulin *et al.*, 2009; Immanuel *et al.*, 2012).

2,4-DAPG-producing fluorescent pseudomonads have been identified in several distinct groups of pseudomonads (Keel *et al.*, 1996; Sharifi-Tehrani *et al.*, 1998; Berg *et al.*, 2002; Mazzola *et al.*, 2004; Naik *et al.*, 2008). Initially, two major phenotypic groups have been distinguished based on the production of antifungal and antibacterial compounds like 2,4-DAPG, hydrogen cyanide and pyoluteorin (Keel *et al.*, 1996). Sharifi-Tehrani *et al.* (1998) suggested that promising biocontrol pseudomonads may be identified, based on their ability to produce 2,4-DAPG, or based on amplified ribosomal DNA restriction analysis (ARDRA) fingerprints. McSpadden Gardener *et al.* (2000) identified 13 and 15 genotypes of Phl-producing pseudomonads by BOX and ERIC-PCR. Picard *et al.* (2000) differentiated 64 RAPD genotype groups among the fluorescent Phl-producing pseudomonads. Frapolli *et al.* (2007) used multilocus sequence typing (MLST) approach to group the worldwide collection of Phl-producing pseudomonads. This led to the identification of six genotypes (A–F) of Phl-producing pseudomonads and proved to be as discriminative as ERIC-PCR and taxonomically correspond to at least six different species. Recently, Phl-producing pseudomonads were assigned to one of these six genotypes (Frapolli *et al.*, 2007; von Felten *et al.*, 2011).

Finger millet [*Eleusine coracona* (L). Gaertner] is cultivated under diverse agro climatic regions of India. It is affected by more than 20 diseases, among which *Pyricularia grisea* is a major pathogen. In India, there are very few reports on the occurrence of Phl-producing pseudomonads in various rhizosphere soils and its biocontrol activity (Velusamy *et al.*, 2006; Naik *et al.*, 2008; Immanuel *et al.*, 2012; Asadhi *et al.*, 2013). Only single genotype of Phl-producing pseudomonads is predominant in the rhizosphere (McSpadden Gardener *et al.*, 2000). So, this study was initiated to explore the Phl-producing pseudomonads genotypes in finger millet rhizosphere by (1) screening for Phl-producing pseudomonads associated with the rhizosphere samples of finger millet; (2) evaluating the antagonistic activity of Phl-producing pseudomonads against phytopathogens and assessing its plant growth-promoting traits; (3) analysing the genotypic diversity of the *phlD* gene through restriction fragment length polymorphism (RFLP) and gene sequencing; (4) identifying the taxonomical position of Phl-producing pseudomonads; (5) delineating Phl-producing pseudomonads genotypes through concatenated sequence analysis.

Materials and methods

Isolation of *Pseudomonas* from rhizosphere soil samples

Rhizosphere soil samples were collected from finger millet fields of Dharmapuri and Villupuram districts of Tamil Nadu, India, and *Pseudomonas* were isolated from these samples by placing *c.* 1 g of soil or roots plus adhering soil into 10 mL of sterilized 1% (v/v) phosphate-buffered saline (PBS). The samples were vortexed for 15 min, then transferred to 70 mL of sterilized 1% (v/v) PBS and incubated on rotary shaker at 150 r.p.m. for 2 h. Samples were serially diluted up to 10^{-6} in 1% (v/v) PBS and plated on King's B agar (KBA) medium (King *et al.*, 1954) in triplicates. After incubation at 28 °C for 48 h, single-colony representatives of different morphological characteristics were isolated and maintained on KBA medium. All the pure cultures were stored at −80 °C in phosphate-buffered 20% (v/v) glycerol.

Detection of Phl-producing pseudomonads

The chromosomal DNA was extracted from all the isolates by the conventional method as described by Rameshkumar & Nair (2009). Screening of Phl-producing pseudomonads was performed by targeting *phlD* gene using specific primer sets as listed in Table 1. DNA of *Pseudomonas protegens* CHA0^T served as positive control, and *Vibrio rhizosphaerae* DSM 18581^T DNA and sterile water served as negative controls. PCRs were carried out in 20 μL reaction mixtures containing 2 μL of 10X PCR buffer, 2 μL of 2.5 mM dNTP mix, 2 μL of each forward and reverse primer (25 ng μL^{-1}), 0.3 μL of Promega Taq DNA polymerase (3 U μL^{-1}) and 2 μL of template DNA (100–200 ng μL^{-1}). The amplification reaction was carried out in an Eppendorf Mastercycler Gradient PCR machine, and the cycling conditions are mentioned in Table 1. After amplification, 3 μL of each PCR products were electrophoresed using 1% (w/v) agarose gel containing 0.25 μg mL^{-1} ethidium bromide (EtBr), and 1 kb DNA ladder (Biotools, Spain) was used as marker. The gel was run using 0.5X TBE buffer at a constant 60 V for 1 h, and the PCR products were visualized using a UV transilluminator.

Exploration of functional traits

Screening for *hcnBC* genes

Hydrogen cyanide (HCN)-coding pseudomonads were identified by amplifying the *hcnBC*-coding genes using specific primer sets as listed in Table 1. DNA of *P. protegens*

Table 1. List of primers used in this study and its PCR conditions

Gene	Primer	Sequence (5′→3′)	PCR programme	Size (bp)	Reference
16S rRNA	fD1 rP2	AGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	Initial denaturation at 95 °C for 5 min was followed by 30 PCR cycles (94 °C for 30 s, 58 °C for 1 min and 72 °C for 60 s) and a final extension at 72 °C (10 min).	1494	Rameshkumar & Nair (2009)
phlD	Phl2a Phl2b	GAG GACGTCGAAGACCACCA ACCGCAGCATCGTGTATGAG	Initial denaturation at 95 °C for 5 min was followed by 30 PCR cycles (94 °C for 30 s, 67 °C for 35 s and 72 °C for 60 s) and a final extension at 72 °C (10 min).	745	Raaijmakers *et al.* (1997).
hcnAB	PM2 PM7-26R	TGCGGCATGGGCGTGTGCCATTGCTGCCTGG CCGCTCTTGATCTGCAATTGCAGGCC	Initial denaturation at 95 °C for 5 min was followed by 30 PCR cycles (94 °C for 30 s, 67 °C for 35 s and 72 °C for 60 s) and a final extension at 72 °C (10 min).	570	Svercel *et al.* (2007)
gyrB	M13R M13 (-21)	CAGGAAACAGCTATGACC TGTAAAACGACGGCCAGT	Initial denaturation at 95 °C for 5 min was followed by 30 PCR cycles (94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s) and a final extension at 72 °C (10 min).	1200	Yamamoto *et al.* (2000)
rpoD	70Fs 70Rs	ACGACTGACCCGGTACGCATGTA ATAGAAATAACCAGACGTAAGTT	Initial denaturation at 95 °C for 5 min was followed by 30 PCR cycles (94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s) and a final extension at 72 °C (10 min).	800	Yamamoto *et al.* (2000)
rpoB	rpoBf1 rpoBr1	CAGTTCATGGACCAGAACAACCCGCT CCCATCAACGCACGGTTGGCGTC	Initial denaturation at 95 °C for 5 min was followed by 30 PCR cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s) and a final extension at 72 °C (10 min).	508	Frapolli *et al.* (2007)

$CHA0^T$ served as a positive control, and *V. rhizosphaerae* DSM 18581^T DNA and sterile water served as negative controls. PCRs were carried out as described in Table 1. After amplification, the PCR products were electrophoresed using 1% (w/v) agarose gel and visualized under UV transilluminator.

Antagonistic activity of Phl-producing pseudomonads

The antagonistic potential of the Phl-producing pseudomonads against blast pathogen *Pyricularia grisea* TN508, wilt pathogen *Fusarium oxysporum* DSM62297 and take all pathogen *Gaeumannomyces graminis* DSM1463, were determined in KBA and modified oat meal agar (MOMA) medium by dual-plate method. A 6-mm plug of actively growing fungal culture was inoculated in the centre of an agar plate and incubated at room temperature (28 ± 2 °C) for 48 h. Later, Phl-producing pseudomonads were inoculated 3.5 cm away from the fungal disc on either side of the plate and incubated at 28 ± 2 °C for 12–14 days. Activity against bacterial black rot pathogen *Xanthomonas campestris* DSM3586 and soft rot pathogen *Erwinia persicina* HMGU155 was assessed by spread-plate method in KBA medium.

Determination of hydrolytic enzymes associated with biocontrol activity

Phl-producing pseudomonads were tested for the production of lytic enzymes like, chitinase, cellulase and protease. Chitinase production was estimated as described by Kole & Altosaar (1985) in Dworkin–Foster (DF) salts minimal medium containing 2.5% (w/v) colloidal chitin. Cellulase activity was determined in carboxymethyl cellulose (CMC) agar containing 5% (w/v) CMC (Sigma Aldrich) (Ariffin *et al.*, 2008). Proteolytic activity was assessed using skimmed milk agar (HiMedia, India) (Wikstrom, 1983).

Evaluation of Phl-producing pseudomonads for PGPR activities

Indole-3-acetic acid (IAA) production by Phl-producing pseudomonads was estimated qualitatively by filter paper overlay method (Bric *et al.*, 1991) in Luria–Bertani (LB)

agar amended with 5 mM L-tryptophan, 0.06% (w/v) sodium dodecyl sulphate and 1% (v/v) glycerol using Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL of distilled H_2O, 7.5 mL of 0.5 M $FeCl_3 \cdot 6H_2O$). The biofilm formation was determined by tissue culture plate (TCP) technique (Christensen *et al.*, 1985), and phosphate solubilization ability of the Phl-producing pseudomonads was assessed in National Botanical Research Institute's (NBRI) phosphate growth medium (Nautiyal, 1999). The ACC deaminase activity was performed as described by Penrose & Glick (2003) using DF salt minimal medium with *P. putida* UW4 TSB30 as positive control. The salt tolerance levels were tested using different concentrations of NaCl, viz. 0.5; 1.0, 1.5 and 2.0 5 mM in Davis' minimal medium and medium without NaCl served as control (Rangarajan *et al.*, 2002).

Detection of 2,4-DAPG

Overnight grown cultures of *Pseudomonas* (OD-0.3 at 600 nm) were inoculated in 100 mL KB medium and incubated at 28 °C at 210 r.p.m. for 48 h. Extraction of 2,4-DAPG was performed in triplicates by modifying the reported protocol (Bonsall *et al.*, 1997). Five millilitre of culture broth containing the bacterial cells was acidified with 10% (v/v) trifluoroacetic acid (TFA) to pH 2.0 and then extracted twice with 10 mL of ethyl acetate by centrifugation at 3500 ***g*** for 6 min (93% extraction efficiency). The ethyl acetate extracts were pooled, evaporated to dryness using a rotary evaporator at 35 °C and then stored at −20 °C until processed. Dried extracts were re-dissolved in 5 mL of 35% (v/v) acetonitrile, filtered through 0.45 μm syringe filters and injected into a Prontosil Spheribond ODS2 column (5 μm, 250 × 4.6 mm, Bischoff Analysentechnik, Germany). The Beckman HPLC system (Beckman Coulter, Germany) consisted of an autosampler 507e with a 10 μL-sample loop: two pumps 114 M, a diode array detector 168 and the chromatographic software GOLD 7.11. Solvent condition included a flow rate of 0.5 mL min^{-1} with a 2-min initialization at 10% (v/v) acetonitrile – 0.1% (v/v) TFA and followed by a 20-min gradient to 100% acetonitrile – 0.1% (v/v) TFA. HPLC gradient profiles were monitored at 270 nm. Standard was prepared using synthetic 2,4-DAPG compound (Chemos, Germany), and four-point standard curves yielded a correlation coefficient of 0.9997.

RFLP analyses of *phlD* gene fragment

The 745-bp *phlD* fragment amplified using primers Phl2a and Phl2b was digested with restriction enzymes HaeIII and TaqI (Thermo Scientific, India). In this study, $CHA0^T$, Pf-5 and Q2.87 were used as comparative strains to represent the genotypes F and C of Phl-producing pseudomonads. Fifteen microliter of the amplified product was digested in a total volume of 40 μL of 1X reaction buffer with 12 U of each restriction enzyme as separate digests. Reaction mixtures were incubated at 37 °C for HaeIII and for TaqI at 60 °C for 4–6 h. Digested products were analysed by electrophoresis on a 2% (w/v) agarose containing 0.25 μg mL^{-1} EtBr, and 1 kb DNA ladder mix (Thermo Scientific, India) was used as marker. The digested products were visualized under a UV illuminator and documented using a Bio-Rad Gel Doc system.

Phylogenetic analysis of *phlD* gene sequence

Based on the RFLP profile of the *phlD* gene, isolates were chosen for *phlD* gene sequence analysis. The amplified *phlD* gene fragment was purified using RBC Bioscience HiYield Gel/PCR fragments extraction kit (Taiwan) according to the manufacturer's instructions. The concentration of the eluted product was estimated, and the purified product was sequenced using Phl2a and Phl2b primers by the dideoxy chain termination method with Big Dye Terminator kit (Applied Biosystems). The reaction products were analysed using capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems). The identities of the sequenced fragments were determined by BLASTN and compared with known sequences. Alignment of *phlD* sequences obtained in this study and those from the databases were performed with CLUSTALW (Thompson *et al.*, 1994). Distance matrices were computed with Kimura 2-parameter using MEGA5.2 (Tamura *et al.*, 2011); phylogenetic trees were constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987), and the topology was checked by bootstrap analysis (1000 data sets).

Molecular evolutionary analysis of 16S rRNA and housekeeping genes

The genomic DNA of Phl-producing pseudomonads was freshly isolated, and 16S rRNA and housekeeping genes were amplified and sequenced using primer sets listed in Table 1. PCRs were carried out in 50 μL reaction mixtures containing 5 μL of 10X PCR buffer, 5 μL of 2.5 mM dNTP mix, 5 μL of each forward and reverse primer (25 ng μL^{-1}), 0.5 μL of Promega Taq DNA polymerase (3 U μL^{-1}) and 5 μL of template DNA (100–200 ng μL^{-1}). The amplification reaction was carried out in an Eppendorf mastercycler gradient PCR machine under the conditions mentioned in Table 1. The amplified samples were loaded on 1% (w/v) agarose gel and resolved at constant voltage of 40 V for 8 h in

0.5X TBE buffer. The products were eluted and purified using RBC Bioscience HiYield Gel/PCR fragments extraction kit (Taiwan) according to the manufacturer's instructions. The concentration of the eluted product was estimated, and the purified product was sequenced through dideoxy chain termination method with Big Dye Terminator kit (Applied Biosystems). The products were analysed using capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems). 16S rRNA gene sequences of Phl-producing pseudomonads were compared with the sequences available from GenBank using BLASTN program and Eztaxon server. 16S rRNA sequences of closely related members were extracted, and 16S rRNA sequences obtained in this study were aligned with CLUSTALW (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the NJ, maximum-likelihood (ML), minimum-evolution (ME) and maximum-parsimony (MP) algorithms available in the MEGA5.2 software (Tamura *et al.*, 2011). The trees were computed using the Kimura 2-parameter method, and topologies of tree were evaluated by bootstrap analysis based on 1000 resamplings.

Concatenated sequence analysis

Sequences of 16S rRNA, *gyrB, rpoD* and *rpoB* were obtained from representative strains of the groups G, H and I Phl-producing pseudomonads, and sequences of other Phl-producing genotypes (A–F) were obtained from the nucleotide database. All the sequences were separately aligned using CLUSTALW (Thompson *et al.*, 1994) and trimmed separately using MEGA5.2 (Tamura *et al.*, 2011). SEQUENCE MATRIX (Vaidya *et al.*, 2011) software was used to create concatenate sequences in the order of 16S rRNA, *gyrB, rpoD* and *rpoB* to assemble and analyse the multi-gene data sets. Concatenated sequences were aligned by CLUSTALW (Thompson *et al.*, 1994), and NJ tree was constructed using the Kimura 2-parameter model for estimating pairwise genetic distances using MEGA5.2 software (Tamura *et al.*, 2011). The degree of statistical support for the nodes on the NJ tree was evaluated by 1000 bootstrap replications.

Results

Isolation and screening of Phl-producing pseudomonads

A total of 500 rhizobacteria with distinct morphological characteristics were isolated from finger millet rhizosphere soil samples, purified and subcultured on KBA medium and stored at −80 °C in phosphate-buffered 20% (v/v) glycerol. The total population of culturable aerobic bacteria associated with the rhizosphere soil was found to be at an average of 4.0 ± 0.2 log CFU g^{-1}. Among the total heterotrophic isolates, only 20 isolates MSSRFD41, MSSRFD68, MSSRFD82, MSSRFD83A, MSSRFD83B, MSSRFD85, MSSRFD86, MSSRFD87, MSSRFD110, MSSRFD114, MSSRFD126, MSSRFD152, MSSRFD254, MSSRFD256, MSSRFD304, MSSRFD398, MSSRFD821, MSSRFD845, MSSRFD846 and MSSRFD865 showed single amplicon of 745 bp fragment size similar to *P. protegens* $CHA0^T$ strain. All the 20 isolates were confirmed as Phl-producing pseudomonads based on the existence of *phlD* gene and phenotypic characteristics, viz. Gram-negative rods, motile, catalase and oxidase positive.

Antagonistic activity of Phl-producing pseudomonads against phytopathogens

Phl-producing pseudomonads exhibited antagonistic activity against fungal pathogens *P. grisea, F. oxysporum* and *G. graminis* in KBA and MOMA medium. Seventeen strains exhibited enhanced activity with zone of inhibition against *P. grisea* (13–20 mm), *F. oxysporum* (7–12 mm) and *G. graminis* (8–16 mm) in KBA. Antagonistic activity of Phl-producing pseudomonads in MOMA media showed wide range of activity against *P. grisea* (8–15 mm), *F. oxysporum* (6–9 mm) and *G. graminis* (6–12 mm). Of the 20 Phl-producing pseudomonads, three isolates MSSRFD152, MSSRFD254 and MSSRFD256 failed to inhibit all the three pathogens in both the media. But all the 20 isolates exhibited prominent antibacterial activity in KBA medium against *X. campestris* DSM3586 and *E. persicina* HMGU155 with inhibiting zones ranging from 15 to 25 mm and 10 to 23 mm, respectively.

Screening for plant growth-promoting activity and hydrolytic enzymes production

A high degree of functional diversity was observed among the pseudomonads isolated from the finger millet rhizosphere, and the functional properties are represented in Table 2. Among 20 Phl-producing pseudomonads, 14 isolates solubilized phosphate in NBRIP agar medium with clearing zones ranging from 3 to 12 mm in size and 13 isolates produced clearing zones (2–8 mm) in chitin-amended agar medium. Eleven isolates exhibited clear zones around the colony in CMC agar medium and 16 displayed proteolytic activity on skim milk agar medium. Only nine isolates were positive for IAA production; 12 isolates produced biofilm and nine produced ACC deaminase. All the Phl-producing isolates grew in 1.0 M NaCl, but only one isolate MSSRF398 survived in 1.5 M NaCl,

Table 2. Plant growth-promoting traits of Phl-producing pseudomonads isolated from finger millet rhizosphere soil

			Antagonistic activity*					PCR†		Hydrolytic enzyme			Plant growth-promoting traits				
S. no	Strain	Phl genotype	*Pg*	*Fo*	*Gg*	*Xc*	*Ep*	*phlD*$^+$	HCN$^+$	Cellulase	Protease	Chitinase	Phosphate	Biofilm	ACC	IAA	Salt tolerance
1.	MSSRFD41	G	+++	++	++	+	+	P	P	P	P	P	P	P	P	P	1.0 M
2.	MSSRFD68	G	++	++	++	+	+	P	P	P	P	P	P	P	P	P	1.0 M
3.	MSSRFD82	G	+	+	+	+	+	P	P	N	P	P	P	N	N	N	1.0 M
4.	MSSRFD83A	G	++	++	++	+	+	P	P	P	P	P	P	P	P	N	1.0 M
5.	MSSRFD83B	G	++	+	++	+	+	P	P	N	N	N	N	N	P	N	1.0 M
6.	MSSRFD85	G	++	+	++	+	+	P	P	P	N	N	P	P	N	N	1.0 M
7.	MSSRFD86	G	+	+	+	+	+	P	P	N	P	P	P	N	N	N	1.0 M
8.	MSSRFD87	G	+++	++	++	+	+	P	P	P	P	P	P	P	P	P	1.0 M
9.	MSSRFD110	G	++	+	+	+	+	P	P	N	P	P	N	N	N	P	1.0 M
10.	MSSRFD114	G	+	+	+	+	+	P	P	N	P	P	P	N	N	N	1.0 M
11.	MSSRFD126	G	+	+	+	+	+	P	P	P	P	P	N	P	N	P	1.0 M
12.	MSSRFD152	G	–	–	–	+	+	P	P	N	N	N	P	N	N	N	1.0 M
13.	MSSRFD304	G	++	++	+	+	+	P	P	P	P	P	P	P	P	N	1.0 M
14.	MSSRFD821B	G	+	+	++	+	+	P	P	N	P	N	N	N	N	P	1.0 M
15.	MSSRFD845	G	++	++	++	+	+	P	P	N	N	N	P	N	N	N	1.0 M
16.	MSSRFD846	G	+++	++	++	+	+	P	P	P	P	P	P	P	P	P	1.0 M
17.	MSSRFD865	G	+++	++	++	+	+	P	P	P	P	P	P	P	P	P	1.0 M
18.	MSSRFD254	H	–	–	–	+	+	P	P	P	P	N	N	P	N	N	1.0 M
19.	MSSRFD256	H	–	–	–	+	+	P	P	N	P	N	N	P	N	N	1.0 M
20.	MSSRFD398	I	++	+	++	+	+	P	P	P	P	P	P	P	P	N	1.5 M

The above-mentioned values are obtained from the biological replicates, and 'P' indicates positive and 'N' for negative.

*Antagonistic activity against *Pg*, *P. grisea*; *Fo*, *F. oxysporum; Gg*, *G. graminis; Xc*, *X. campestris; Ep*, *E. persicina,* and range of inhibition '+++' indicates > 15 cm, '++' indicates inhibition zone < 15 cm, '+' indicates inhibition zone < 8 cm, '–' indicates no inhibition.

†PCR detection of genes encoding the production of DAPG and HCN.

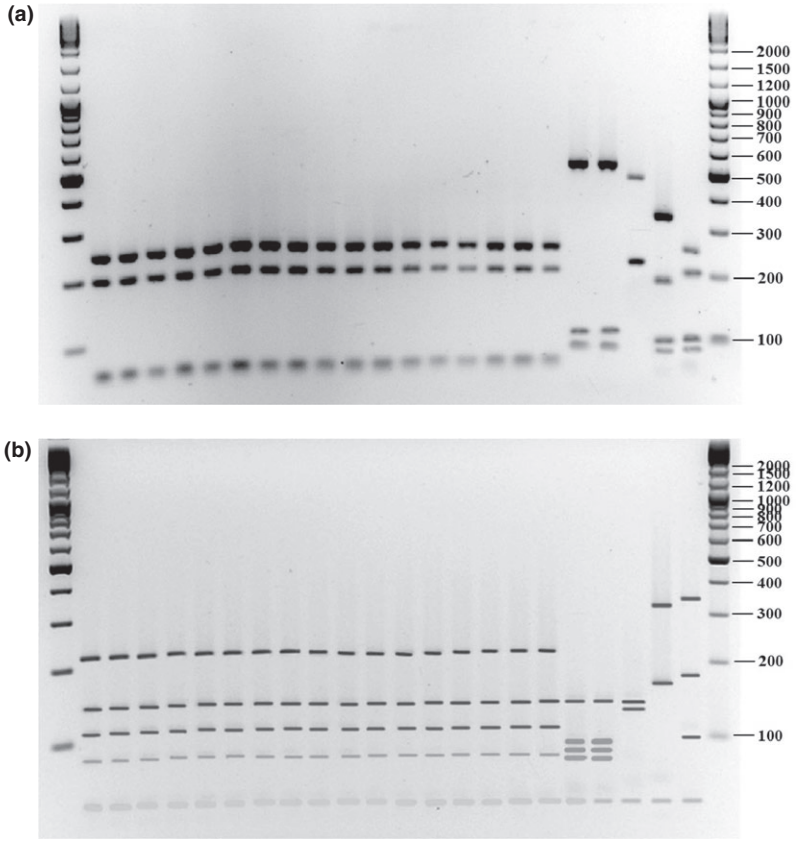

Fig. 1. (a and b) RFLP analysis of *phlD* gene amplified with phl2a and phl2b. The 745 bp of the *phlD* gene digested with TaqI (a) and HaeIII (b). Lanes 1 and 24: 1 kb ladder; Lane 2-MSSRFD41; 3-MSSRFD68; 4-MSSRFD82; 5-MSSRFD83A; 6-MSSRFD83B; 7-MSSRFD85; 8-MSSRFD86; 9-MSSRFD87; 10-MSSRFD110; 11-MSSRFD114; 12-MSSRFD126; 13-MSSRFD152; 14-MSSRFD304; 15-MSSRFD821; 16-MSSRFD845; 17-MSSRFD846; 18-MSSRFD865; 19-MSSRFD254; 20-MSSRFD256; 21-MSSRFD398; 22-Q2.87 and 23-CHA0^T. Five distinct Phl-producing pseudomonads genotypes were identified that corresponded to the previously characterized C and F genotypes of Phl-producing pseudomonads as well as three new genotypes designated G, H and I.

and none of the isolates were able to grow in 2.0 M NaCl.

RFLP analyses of *phlD* gene

The RFLP fingerprinting pattern of the Phl-producing pseudomonads were compared with isolates of F and C genotypes. The amplification of *phlD* gene using specific primers Phl2a and Phl2b yielded a 745-bp amplicon in all the 20 Phl-producing pseudomonads. Restriction digestion of the amplified product with TaqI and HaeIII gave polymorphic banding patterns which clearly differed in size compared to the fragments of genotypes F and C. Based on these polymorphisms, the isolated Phl-producing pseudomonads were assigned to new genotypes G, H and I (Fig 1a and b) in continuation to the already reported genotypes of A–F (Frapolli *et al.*, 2007). Among the three genotypes, G was the largest group with 17 pseudomonads, followed by H with 2 and I having 1 Phl-producers.

Sequence analysis and phylogenetic divergent of *phlD* gene

Totally 13 representative Phl-producing pseudomonads from each genotype based on functional traits were chosen for the *phlD* gene sequencing. The *phlD* gene sequences obtained were in an average read length of 690 bp, and sequences showed maximum similarity of < 87–89% with deposited sequences of *phlD* gene which codes for known protein polyketide synthases, especially with *P. fluorescens phlD* gene. Phylogenetic analysis of *phlD* gene showed a divergent relationship among the isolates from this study, as well as with the existing *phlD* genotypes. The evolutionary analyses revealed clear discrimination between the genotypes from this study (G-I) and existing (A–F) Phl-producers. *phlD* gene sequences from this study were found to cluster in a monophyletic clade associated with high bootstrap support values (Fig. 2) which clearly indicated the existence of new genotypes among Phl-producing pseudomonads.

Phylogenetic analysis of Phl-producing pseudomonads

The taxonomical position of 20 Phl-producing pseudomonads was determined based on partial 16S rRNA gene (*c.* 1400 bp) sequencing, and the results were compared with the type strains 16S rRNA sequences and other sequences in the databases. Similarity index analysis indicated that 17 isolates (genotype – G) showed 98–99%

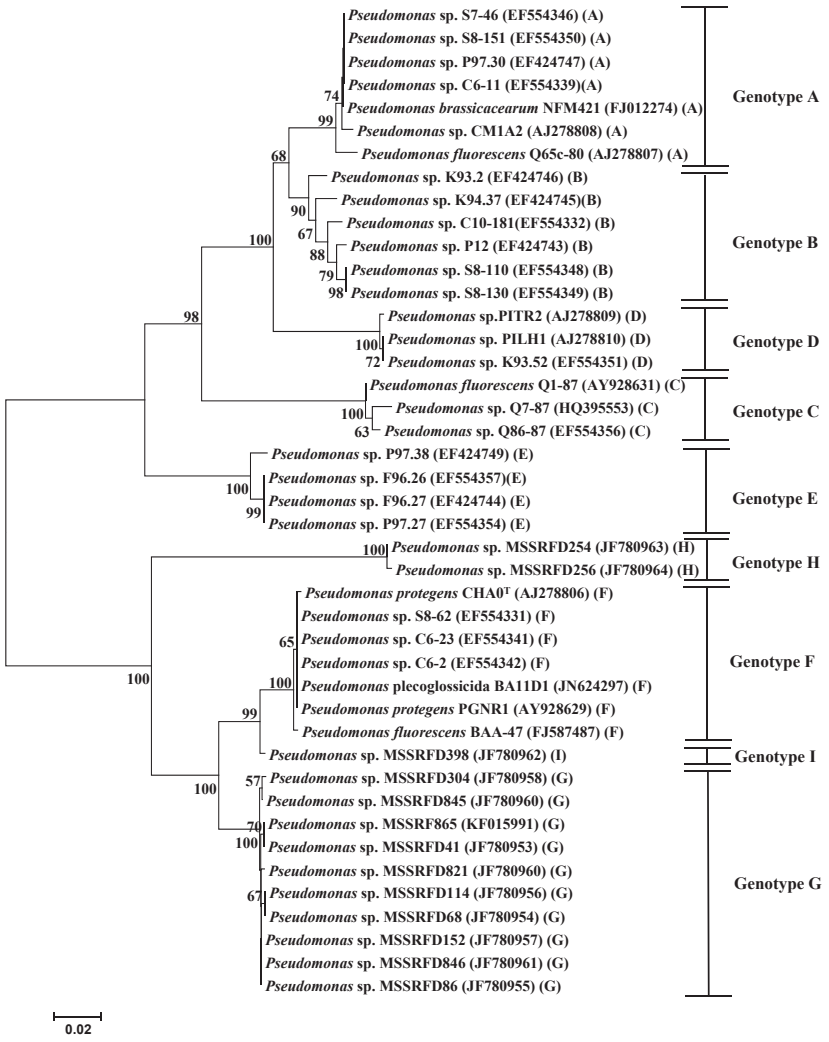

Fig. 2. Phylogenetic tree based on the comparison of *phlD* gene sequence from this study with the reported A–F genotype sequences retrieved from the NCBI database inferred using the NJ method. Numbers at the nodes are percentage bootstrap values based on 1000 resampled data sets with only bootstrap values ≥ 50% shown.

similarity to *P. putida* group strains (*sensu strictu*) like *P. taiwanensis* BCRC 17751^T, *P. plecoglossicida* FPC 951^T, *P. mosselii* CIP 105259^T and *P. monteilii* CIP 104883^T. The other two genotypes of Phl-producing pseudomonads belonged to the group of *P. aeruginosa* (*sensu strictu*). Genotype H showed maximum similarity index of 99.2% to *P. alcaligenes* LMG 1224^T, and 98% to *P. aeruginosa* LMG 1242^T and *P. resinovorans* LMG 2274^T. Genotype I showed 98.9% similarity index to *P. mendocina* LMG 1223^T and *P. oleovorans* DSM 1045^T. The evolutionary distance for 20 Phl-producing pseudomonads and related type strains was computed using the Kimura 2-parameter method and was in the units of the number of base substitutions per site. Their evolution was inferred using the NJ method (Fig. 3) and grouped based on Anzai *et al.* (2000). Even though 17 (genotype G) isolates displayed similarity index of 98–99% to existing type strains in *P. putida* group (*sensu strictu*), the result of phylogenetic analysis revealed a separate clade from *P. putida* group (*sensu strictu*). Based on bootstrap analysis of 1000 resampled data sets, only values above 55% are shown. The constructed ML, ME and MP trees strongly supported the topological position of these Phl-producing pseudomonads (data not shown). Two isolates MSSRFD254 and MSSRFD256 (genotype H) also formed an outward phylogenetic relationship with *P. alcaligenes* LMG 1224^T and MSSRFD398 (genotype I) clade with the *P. mendocina* LMG 1223^T. Phylogenetic analysis showed that the isolates in genotype G and I formed an outward clade from its closest type strains. Among the 17 isolates of genotype G, the existence of intrageneric diversity was clearly represented in the phylogenetic tree (Fig. 3). Similarly, 16S rRNA phylogenetic tree constructed with genotypes of Phl-producing pseudomonads, and closest strains clearly differentiated the new genotype G, H and I (Supporting Information, Fig. S1).

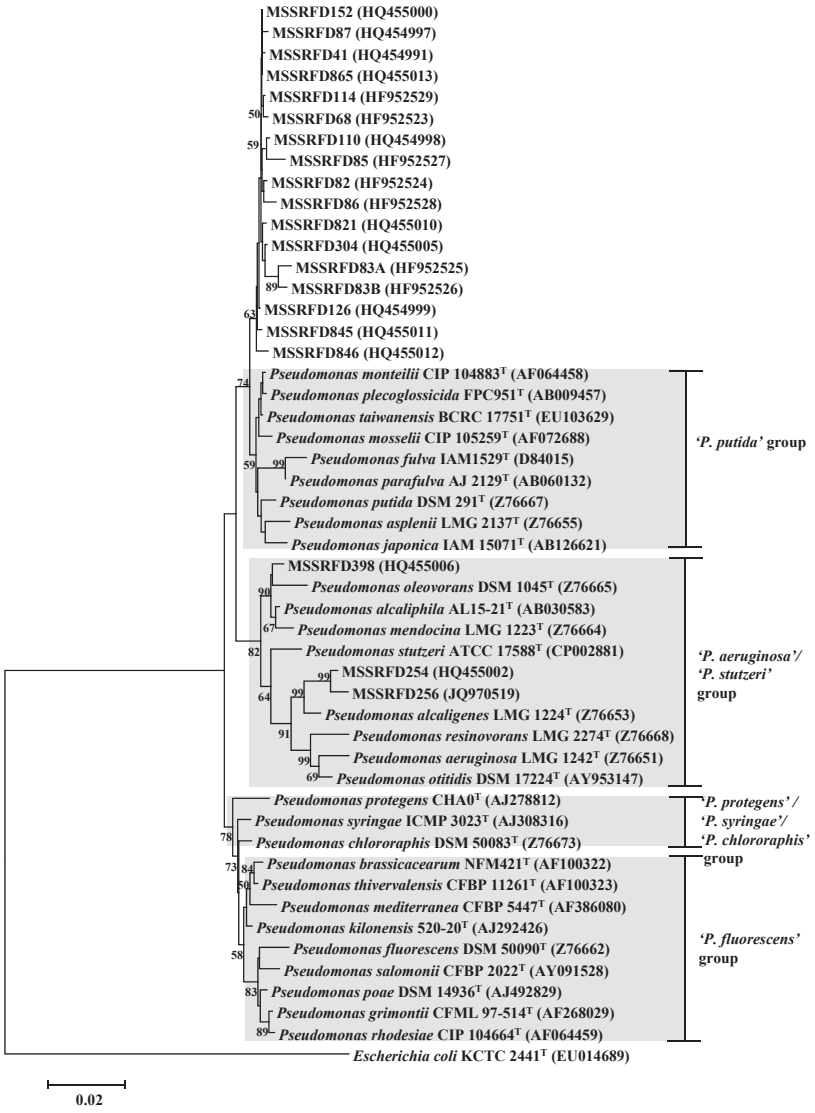

Fig. 3. Phylogenetic relationship of the Phl-producing pseudomonads to related type strains of *Pseudomonas* species based on 16S rRNA gene sequences. The tree was constructed by the NJ method and computed using the Kimura 2-parameter method. Bootstrap percentages above 50 are given at branching points. The optimal tree with the sum of branch length = 0.38498299 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches and only bootstrap values ≥ 50% shown. *Escherichia coli* KCTC 2441^T was used as an out-group.

Molecular evolutionary analysis of *gyrB, rpoD* and *rpoB* genes

Three isolates from genotype G (MSSRFD41, MSSRFD304 and MSSRFD865), two from genotype H (MSSRFD254 and MSSRFD256) and one from genotype I (MSSRFD398), were chosen for sequence analysis of their housekeeping genes *gyrB, rpoD* and *rpoB*. Genotype G *gyrB* and *rpoB* gene sequence analyses showed highest similarity with *P. protegens* Pf-5 (95%, 94%) and *P. protegens* CHA0^T (94%, 93%); similarity level of *rpoD* was 90% to *P. protegens* Pf-5 and *P. protegens* CHA0^T, and the similarity index of these isolates to other *Pseudomonas* spp. was < 90–92%. Sequence analyses of Genotype H showed *rpoB* (94%), *gyrB* (90%) and *rpoD* (88%) similarity to *P. mendocina* LMG 1223^T, *P. otitidis* DSM 17224^T and *P. alcaligenes* LMG 1224^T. The genotype I exhibited 98% similarity to *P. mendocina* LMG 1223^T for all the three genes. Based on the similarity level and the phylogenetic tree analysis, genotype G displayed separate topological position from other *Pseudomonas* spp. for housekeeping genes *gyrB* (Fig. S2), *rpoD* (Fig. S3) and *rpoB* (Fig. S4). The sequence analyses of housekeeping genes and 16S rRNA gene revealed similar taxonomical clarifications of all the three genotypes of Phl-producing pseudomonads. The phylogenetic analysis of all the three housekeeping genes of the genotype G and I formed an outward clade which supported the results of 16S rRNA analysis.

Concatenated sequence analysis

Partial sequences of 16S rRNA, *gyrB, rpoD* and *rpoB* genes from genotypes G, H and I; 57 Phl-producing

pseudomonads genotypes A–F and 14 neighbouring pseudomonads were used for the concatenation. The alignment and trimming of the sequences individually yielded concatenated of 2969 nucleotides (16S rRNA-1376; *gyrB*-533; *rpoD*-608 and *rpoB*-452 bp). Results of concatenated sequence analysis strongly indicated that these three genotypes G, H and I from this study could be a new genotype among the reported Phl-producing pseudomonads genotype (A–F). The phylogenetic tree based on the concatenated sequences confirmed the clustering of the genotypes G, H and I isolates with high bootstrap value and formed a separate clade from the currently known genotypes (A–F) of Phl-producing pseudomonads (Fig. 4).

Quantification of DAPG production

2,4-DAPG production by all the 20 Phl-producing pseudomonads was quantified by HPLC analysis and compared to the standard. 2,4-DAPG eluted at a retention time of 16.6 min. Average amount of 2,4-DAPG production in genotype G (16 isolates) was in the range of 35–40 $\pm$ 1 μg mL^{-1}, and one isolate (MSSRF152) produced 6 $\pm$ 0.9 μg mL^{-1}. Genotype H-(2 isolates) produced 7 $\pm$ 0.7 μg mL^{-1} of 2,4-DAPG and genotype I-(1 isolate) produced 13 $\pm$ 0.9 μg mL^{-1}.

Overall, RFLP, phylogenetic and concatenated sequence analysis clearly differentiated the genotypes G, H and I which differed from earlier reported Phl-producing pseudomonads genotypes A–F. In particular, genotype G showed the most distinct clade when compared to other species of pseudomonads. These analyses visibly indicated the existence of novel and distinct species among the pseudomonads population. This study suggests that finger millet rhizosphere harbours significant populations of Phl-producing pseudomonads which can protect the plant from phytopathogenic infections and enhance plant growth.

Discussion

The primary objective of this research was to explore the genetic and functional diversity of Phl-producing pseudomonads community from the finger millet rhizosphere. In many studies, *phlD* gene was used as a marker for the isolation of Phl-producing pseudomonads (McSpadden Gardener *et al.*, 2000, 2005; Mavrodi *et al.*, 2001; Picard & Bosco, 2003; De La Fuente *et al.*, 2006; von Felten *et al.*, 2011; Immanuel *et al.*, 2012). The present study also proved that the *phlD* gene is highly conserved among pseudomonads and could be used as a reliable marker to identify Phl-producing pseudomonads. Isolation of Phl-producing pseudomonads from different plant rhizospheres has been reported all over the world (Keel *et al.*, 1996; McSpadden Gardener *et al.*, 2000; De La Fuente *et al.*, 2006; Velusamy *et al.*, 2006; Immanuel *et al.*, 2012). In this study, the diversity and taxonomic position of the Phl-producing pseudomonads were analysed from finger millet rhizosphere soil samples of Tamil Nadu, India. The results indicated that new genotypes of *Pseudomonas* spp. recovered from the finger millet rhizosphere soil samples harboured *phlD*-coding gene and possessed potential PGPR traits such as production of hydrolytic enzymes, phosphate solubilization, ACC deaminase production, biofilm formation as reported earlier (Chernin & Chet, 2002; Haas & Defago, 2005; Naik *et al.*, 2008). But in this study, phosphate solubilization was observed in the groups of *P. putida* and *P. aeruginosa* (*sensu strictu*), and these results contradict to the findings of Browne *et al.* (2009) who demonstrated that *Pseudomonas* strains with the ability to solubilize phosphate were linked to a single phylogenetic lineage within the *P. fluorescens* group.

Some of the Phl-producing pseudomonads failed to inhibit the phytopathogens under *in vitro* conditions, it may be due to the impact of factors like medium, concentration of metabolite produced and expression of antibiotic coding genes. Similarly, Keel *et al.* (1992) and McSpadden Gardener *et al.* (2005) reported that the production of 2,4-DAPG was media dependent and required suitable environments to inhibit phytopathogens. Paulin *et al.* (2009) showed the expression of *phlD* gene and the production of 2,4-DAPG significantly changed over time, which was also influenced by the presence of the pathogen, growth medium and was a time-dependent response. These results are clearly in line with the earlier findings, indicating that the expression of the functional genes or production of DAPG purely depends on the nutrient conditions. Hence, it is essential to understand the diversity of *phlD* gene and the existence of predominant Phl-producing *Pseudomonas* genotypes in the rhizosphere (McSpadden Gardener *et al.*, 2005) to evaluate the potential or frequency of horizontal transfer of the biosynthetic genes between members of the rhizosphere microbial community and their role in biocontrol.

In many studies, diversity of *phlD* gene has been explored through different markers like RFLP (Mavrodi *et al.*, 2001; Picard & Bosco, 2003), BOX and ERIC-PCR (McSpadden Gardener *et al.*, 2000), ARDRA (Keel *et al.*, 1996; Sharifi-Tehrani *et al.*, 1998; McSpadden Gardener *et al.*, 2000) and RAPD (Picard *et al.*, 2000). Mavrodi *et al.* (2001) clearly showed the diversity of *phlD* gene by RFLP analysis that correlated closely with clusters defined previously by BOX PCR genomic fingerprinting, indicating the usefulness of *phlD* as a marker of genetic diversity and population structure among 2,4-DAPG producers. Among these, RFLP analysis of the *phlD* gene using

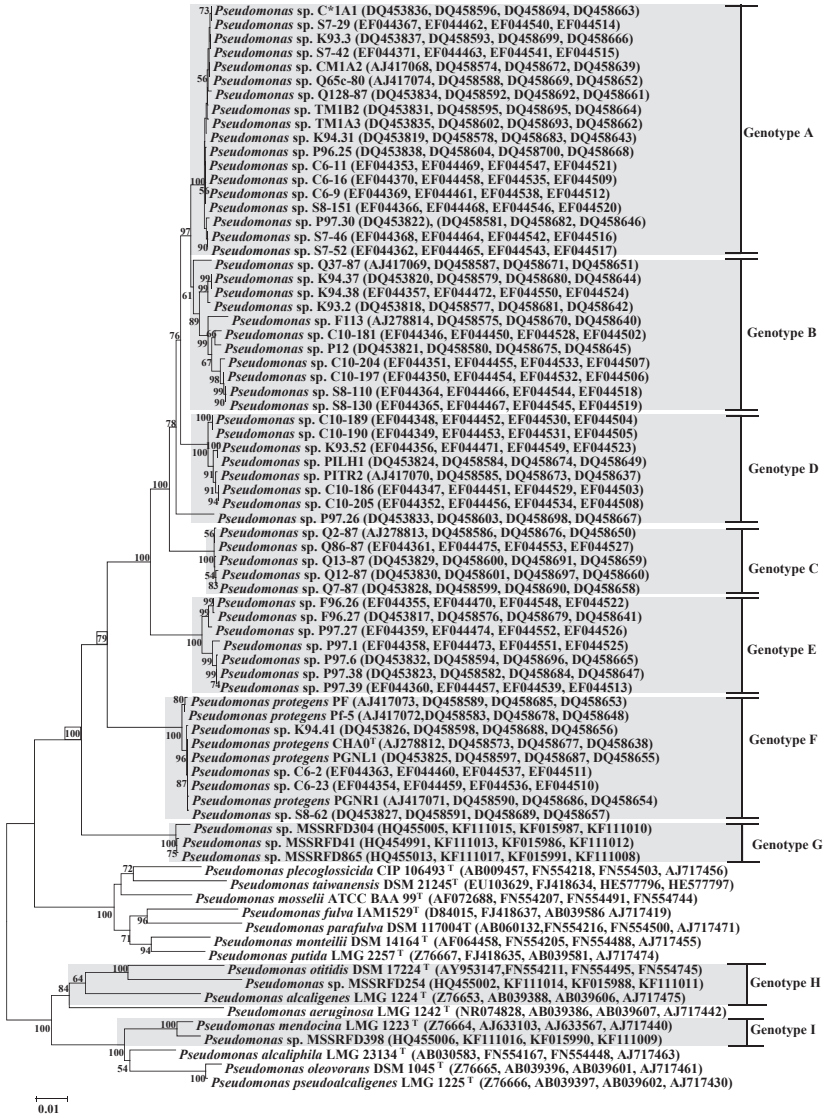

Fig. 4. NJ phylogenetic tree based on concatenated sequences 2969 bp of the 16S rRNA (1376), *gyrB* (533), *rpoD* (608) and *rpoB* (452), showing the position of the outward group as well as clear discrimination of Phl-producing pseudomonads. The stability of the grouping was estimated by bootstrap percentages from 1000 replicates, and only bootstrap values ≥ 50% were shown. Similar tree topology was obtained with the ML algorithm. Numbers at the nodes show the percentage bootstrap values and the optimal tree with the sum of branch length = 0.74772799.

HaeIII has been widely used for analysing the ecology and population genetics of this beneficial group of soil bacteria at different levels (Mavrodi *et al.*, 2001; Picard & Bosco, 2003; McSpadden Gardener *et al.*, 2005). In our study, digestion of *phlD* gene with HaeIII enzyme showed high polymorphism among the Phl-producers when compared to digestion with TaqI which was in concurrence with the findings of Mavrodi *et al.* (2001). This clearly shows that enzyme HaeIII can be used to study the population structure of Phl-producing genotypes from different groups of pseudomonads. DGGE and quantitative PCR were used to assess *phlD* gene diversity and biocontrol efficiency of Phl-producing pseudomonads (Bergsma-Vlami *et al.*, 2005; Frapolli *et al.*, 2010; von Felten *et al.*, 2011; Almario *et al.*, 2013; Asadhi *et al.*, 2013).

Frapolli *et al.* (2007) systematically analysed the six reference genotypes (A–F) of Phl-producing pseudomonads and recommended MLST approach to study the genetic and evolutionary relations among Phl-producing pseudomonads. In our studies, concatenated sequence approach clearly resolved the phylogeny and population structure of the six reference genotypes (A–F) and three new genotypes (G, H and I). Concatenated sequence of the 16S rRNA and the housekeeping genes showed a clear taxonomical position of genotype G which formed a distinct clade from *P. putida* (*sensu strictu*) and genotype H formed an outward lineage with *P. alcaligenes* LMG 1224^T. Currently, concatenated sequences of multiple gene approaches have been reported as a rapid and robust classification method for the microbial molecular

systematics (Gevers *et al.*, 2005) and to demonstrate variable levels of genetic diversity and recombination (Curran *et al.*, 2004). 16S rRNA sequence analysis showed that Phl-producing genotypes A–E belong to the *P. fluorescens* complex (Frapolli *et al.*, 2007). But, group F was defined as a separate group of Phl-producing pseudomonads (ARDRA 1 group) by Keel *et al.* (1996) and does not belong to the *P. fluorescens* complex as shown by Frapolli *et al.* (2007).

Earlier studies have reported the presence of *phlD* gene more frequently among *P. fluorescens* (Kole & Altosaar, 1985; Bangera & Thomashow, 1999), *P. aeruginosa* (Naik *et al.*, 2008) and *P. putida* (Berg *et al.*, 2002; Mazzola *et al.*, 2004), but not among the closest strains of *P. alcaligenes* and *P. mendocina.* Probably, genotypes G and H can be novel species, as they indicate new genotypes among Phl-producing pseudomonads and formed a distinct taxonomical clade from *P. putida* and *P. aeruginosa* (*sensu strictu*) groups. Recently, Lopez *et al.* (2012) reported a novel species of *P. baetica* LMG 25716^T within the group of *P. fluorescens* with high similarity of 99.4–99.3% with type strains. Although in this study, the genotypes of G and H exhibited high similarity index of 98.0–99.2% to closely related type strains, they formed an outward clade in all phylogenetic analysis and functionally these genotypes exhibited unique properties.

Through our analysis, all the methods visibly proved a high degree of discrimination among the *phlD* gene population structure. This is in agreement with McSpadden Gardener *et al.* (2000) who stated that in spite of multiple genotypes being isolated, only one genotype was predominant in the rhizosphere. In the present study, also a single genotype of Phl-producing pseudomonads was found to be predominant in the finger millet rhizosphere soil. So, *phlD* gene can be used as a marker gene to study the genetic diversity and the population structure of Phl-producing pseudomonads from rhizosphere soils of different geographical origins. Initially, Keel *et al.* (1996) showed two groups of antibiotic-producing pseudomonads through ARDRA analysis; subsequently, these two groups were delineated, substantial diversity was found and the number has increased as a result of differences in genotypic and phenotypic traits (Frapolli *et al.*, 2007). Fluorescent pseudomonads (Keel *et al.*, 1992; Picard & Bosco, 2003; Mazurier *et al.*, 2009) are dominant DAPG-producing groups and have been reported to act as potential biocontrol agents mainly against fungal pathogens (Kole & Altosaar, 1985; Haas & Defago, 2005). Interestingly, in this study, Phl-producing pseudomonads were observed in groups of *P. putida* and *P. aeruginosa* (*sensu strictu*) and not in *P. fluorescens.* The existence of *phlD* gene in these groups may have occurred due to horizontal gene transfer or evolutionary changes in the *phlD* biosynthetic plant chalcone synthase genes, which belongs to type III polyketide synthase gene and is known to be unusual in prokaryotes (Bangera & Thomashow, 1999). So, there is the possibility of a common evolutionary origin, probably as a result of horizontal gene transfer from plants to the bacteria (Cook *et al.*, 1995). This hypothesis is on par with the fact that bacterial transformation could take place inside the plant tissues (Bertolla *et al.*, 1999). The evolutionary relationship of *phlD* gene among these Phl-producing pseudomonads needs to be studied to further understand the evolution of this gene.

The diversity of *phlD* gene has been studied from different rhizospheres, *viz.* wheat (McSpadden Gardener *et al.*, 2000), rice (Immanuel *et al.*, 2012) and maize (Picard & Bosco, 2003). This is the first study to systematically investigate the distribution of Phl-producing pseudomonads associated with the finger millet rhizosphere soils and has proved the existence of predominant Phl-producing pseudomonad genotype. Based on the results of RFLP, concatenated sequences and *phlD* gene sequence analysis, it is proposed that the genotypes G, H and I could be considered as new genotypes and the isolates of genotypes G and H may belong to new phylogenetic group as it forms an outward clade in all the sequence analyses. The knowledge of the *phlD* gene diversity sets a baseline to investigate the relationships between Phl-producing pseudomonads and their impact on the biocontrol of phytopathogens and plant growth.

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Statement

The GenBank accession numbers for 16S rRNA HQ454991, HQ454997, HQ454998, HQ454999, HQ455000, HQ455002, HQ455005, HQ455006, HQ455010, HQ455011, HQ455012, HQ455013 and JQ970519; *phlD* JF780953 to JF780964 and KF015991, *gyrB* KF111013–KF111017, *rpoB* KF111008–KF 111012 and *rpoD* KF015986- KF015990.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Neighbor-joining phylogenetic tree inferred from 16S rRNA sequences of Phl-producing pseudomonads groups and representative of closest phl-producing pseudomonads species.

Fig. S2. Neighbor-joining tree of Phl-producing pseudomonads group A-I and type strains of *Pseudomonas* species inferred from *gyrB* DNA sequences.

Fig. S3. The evolutionary history of *rpoD* DNA sequences among the groups of Phl-producing pseudomonads and type strains of *Pseudomonas* species was inferred using the Neighbor-Joining method.

Fig. S4. Neighbor-joining tree of Phl-producing pseudomonads group A-I and type strains of *Pseudomonas* species inferred from *rpoB* DNA sequences.