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Molecular identification and assessment of genetic diversity of fluorescent pseudomonads based on different polymerase chain reaction (PCR) methods

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A total of 66 fluorescent pseudomonads strains isolated from diverse Tunisian environmental biotypes (wastewater, compost, wastewater treatment plant, etc.) were analysed by two polymerase chain reaction (PCR)-based methods, 16S-23S intergenic spacer regions (ITS)-PCR and repetitive extragenic palindromic (BOX)-PCR. These typing techniques were evaluated to assess their usefulness as tool to study the *Pseudomonas* diversity within this complex group. Genetic analysis using ITS- and BOX-PCR generated respectively 12 and 45 distinct profiles. Phylogenetic relationships within fluorescent pseudomonads were examined by analyzing partial 16S rRNA and *rpoB* genes sequences. The phylogenetic resolution of the rpoB tree was higher than that of the 16S rRNA tree. Moreover, the sequencing of the rpoB gene has recognized 13 different species and sub-species, while the 16 rRNA gene sequencing differentiated only 9 species.

Key words: Fluorescent pseudomonads, 16S-23S intergenic spacer regions (ITS)-PCR, repetitive extragenic palindromic (BOX)-PCR, 16S rRNA, *rpoB*.

INTRODUCTION

The genus *Pseudomonas*, firstly described by Migula in 1894, is characterized as straight or slightly bent Gram negative rods with one or more polar flagellae, not forming spores (Fuchs et al., 2001). Its metabolism is chemoorganotrophic and strictly aerobic with a respiratory type in which oxygen is used (Fuchs et al., 2001).

Pseudomonas "sensu stricto" group I is the largest of the groups, and includes both fluorescent and non fluorescent ones. The most important fluorescent species are *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida* and plant pathogenic species (*Pseudomonas syringae*) (Scarpellini et al., 2004). Several species of rRNA group I pseudomonads have the ability to produce and excrete, under condition of iron limitation, soluble yellow green pigments that fluorescence under UV light (Bultreys et al., 2003), named pyoverdines (PVDs) or pseudobactins, which act as siderophores for these bacteria (Meyer, 2000). These molecules are thought to be associated with biocontrol of fungal pathogens in the biosphere (Fuchs et al., 2001).

The abundance of literature on genus *Pseudomonas*, is due to their elevated metabolic versatility capable of utilizing a wide range of simple and complex organic compounds and holding an important position in biosphere ecology (Scarpellini et al., 2004). Consequently, they are isolated from a variety of natural sources including soil, plants and mineral waters and

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from clinical specimens and they are characterized by a high level of metabolic diversity (Moore et al., 1996). Often, they are able to survive and multiply in poor nutrient conditions (Moreira et al., 1994).

Fluorescent pseudomonads have been considered as an important bioinoculants due to their innate potential to produce plant growth promoting hormones (Latour et al., 2003) and antimicrobial secondary metabolites (Costa et al., 2006; Dong and Zhang, 2005).

Considering the multiple applications of fluorescent pseudomonads, it is essential to study their diversity, which will be useful in designing strategies to use these strains as bioinoculants.

The characterization of *Pseudomonas* genus is faced with difficulties based on their genetic heterogeneity. Recently, the development of molecular techniques has yielded innovative alternative tools for demonstrating the mechanisms underlying biocontrol properties (Massart and Jijakli, 2007) and understanding the role of these bacteria in bioremediation, plant spoilage and pathogenicity (Ravi Charan et al., 2011).

Microbial DNA fingerprinting has been reported extensively using polymerase chain reaction (PCR)-based techniques such as ITS- and BOX-PCR. The 16S-23S polymorphism has been used to differentiate among Gram negative bacteria at the species as well as the genus level and to assess the phylogenetic closeness among bacteria (Ouzari et al., 2008; Wang and Jayarao, 2001). Other methods appeared interesting for the delineation of species, subspecies (Louws et al., 1998) or pathovars (Louws et al., 1994) now available. Box-PCR is the most commonly used technique due to its simplicity, efficiency and low cost (Brusetti et al., 2008). This is a particular version of repetitive extragenic palindromic-PCR (rep-PCR) that uses the BOX-A1R primer (Versalovic et al., 1991). BOX-PCR, has revealed the possibility of delineating *P. syringae* genomospecies (Marques et al., 2000), as well as for typing *Aeromonas* spp. strains (Tacao et al., 2005).

On the other hand, the 16S rRNA and *rpoB* gene sequences have been widely used to assess phylogenetic closeness among bacteria. In many cases, the 16S rRNA gene sequence is not sufficiently discriminative for taxonomic purposes (Adékambi et al., 2009). The high degree of conservation of *rrs* gene led to a small number of informative sites in its sequence (Ait Tayeb et al., 2005) and it fails to reveal precise and statistically supported phylogeny at the species level (Hasegawa and Hashimoto, 1993). Consequently, several attempts have been made to identify other genes, which can be used to determine the relatedness between species, such as *gyrB*, *rpoD*, *ampC*, *oprI*, *pilA*, etc. for *Pseudomonas* genus (Bennasar et al., 2010). The *rpoB* gene, which codes for the RNA polymerase beta subunit, has been used as signature for bacterial identification for phylogenetic analysis (Ait Tayeb et al., 2005).

The aim of this investigation was to distinguish the different haplotypes of fluorescent *Pseudomonas* collection by combining two PCR-based molecular methods. In addition, we identified representatives of the abundant groups by 16S rDNA and *rpoB* sequencing.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas strains described in the present study were collected over a period of 18 months from environment and clinical origin. Environmental samples were transported to the laboratory in sterile stomacher bags, stored at 4°C, and analyzed within 24 h. Strains whose designations begin respectively with PsWw, PsWs and PsWt; were collected from water (waste water, sea water and thermal water); strains whose designations begin with PsS were collected from soil and strains whose designations begin respectively with PsC and PsTp were isolated from compost and waste water treatment plant. Clinical strains are designed as PsCL.

Isolation and growth conditions

Ten grams or 10 ml of sample were suspended in 90 ml sterile distilled water. Fifty microliters of an appropriate serial dilution was spread on King's B (KB) and King's A (KA) agar medium (Difco Laboratories), and one of each of the phenotypically different colonies developing a fluorescent halo after 48 h of incubation at 25°C was further purified by streaking it on the same medium (Munsch et al., 2000). Routine growth was in Luria-Bertani (LB) medium or King's B liquid medium (King et al., 1954).

For this study, putative *Pseudomonas* colonies were collected from representative plates and initially characterized by Gram staining and testing for oxidase and catalase activities. All single colonies that were confirmed as putative *Pseudomonas* spp. (Gram negative, oxidase positive and catalase positive) were used for further characterization as described below. Strains were preserved by mixing overnight LB culture with 50% glycerol (1:1, vol/vol) and storage at -80°C.

The fluorescence was detected in the iron-poor liquid growth medium (Meyer et al., 2002), the casamino acid (CAA) medium, consisting of (per liter) 5 g of low-iron Bacto casamino acid (Difco), 1.54 g of $K_2HPO_4 \cdot 3H_2O$ and 0.25 g of $MgSO_4 \cdot 7H_2O$.

Genomic DNA extraction

A single bacterial colony was inoculated into 5 ml (LB) and grown for 16 h at 30°C. Saturated culture was harvested with centrifugation for 3 min at 12,000 rpm. The cell pellet was resuspended and lysed in 200 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µl of 5M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12,000 rpm for 10 min. An equal volume of chloroform was added to the clear supernatant. Following centrifugation at 12,000 rpm for 3 min, the extract supernatant was precipitated with 100% EtOH, washed twice with 70% EtOH, dried and redissolved in 50 µl 1x TE buffer (Chen and Kuo, 1993).

PCR amplification

All the PCR (except BOX-PCR) were performed in a volume of 50 µl of bacterial genomic DNA solution, 10 µl of 5 × PCR buffer, 100

µM of each dNTP, 1 mM of $MgCl_2$, 0.25 µM of each primer and 1 U of *Taq* Polymerase.

Polymorphisms were sought in the 16S-23S spacer region of the rRNA genes by DNA amplification using the primers complimentary to the conserved regions of the 16S and 23S bacterial rRNA genes. DNA 16S–23S intergenic spacer (ITS) region amplification was performed using the primer set 16F945 and 23R458 (16F945 5'-GGGCCCGCACAAGCGGTGG-3'; 23R458 5'-CTTTCCCTCACGGTAC-3') (Lane et al., 1985) and the following thermal profile: 4 min at 94°C; 40 cycles consisting of 94°C for 30 s, 45°C for 30 s, 72°C for 45 s; final extension of 72°C for 7 min.

BOX-PCR was performed as described by Brusetti et al. (2008). Mixtures contained 1× PCR buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.8 µM of BOX-A1R primer, 5% of dimethylsulfoxide, 1.3 U of Taq DNA polymerase and standardized 15 ng of genomic DNA in a final volume of 30 µl. Reactions were denatured at 94°C for 5 min, subjected to 35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min.

The discriminatory power of ITS- and BOX-PCR methods was calculated by application of Simpson numerical index of diversity (Hunter and Gaston, 1988).

DNA 16S region amplification was performed using selective primers to amplify a 969-bp fragment: forward primer Ps-for (20-mer [5'-GGTCTGAGAGGATGATCAGT-3']) and reverse primer Ps-rev (18-mer [5'-TTAGCTCCACCTCGCGGC-3']) (Widmer et al., 1998). The PCR program used was: 4 min at 94°C; 30 cycles consisting of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s; final extension of 72°C for 7 min; and final cooling at 16°C.

RpoB region amplification was carried out using selective primers to amplify a 1247-bp fragment: LAPS (5'-TGGCCGAGAACCAGTTCCGCGT-3') and LAPS-27 (CGGCTTCGTCCAGCTTGTTCAG) (Ait Tayeb et al., 2005). The PCR program used was: 94°C for 4 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 7 min.

PCR amplifications were performed with a Biometra UNOII Thermal Cycler. 5 µl of PCR products were analysed by electrophoresis at 100 V respectively on (2, 2, 1.5 and 1%) agarose gels for ITS-PCR, BOX-PCR, 16S rDNA and *rpoB,* containing 0.2 µg of ethidium bromide ml^{-1} in TAE buffer and photographed using UV light.

Partial sequencing of the 16S-rDNA and rpoB

PCR products obtained from representative BOX and ITS groups (34 bacterial isolates) were purified with a Promega PCR purification Kit, and sequenced using an Applied Biosystems sequencer. Sequences of the PCR products were aligned and corrected manually with Chromas Pro (version 1.34). The BLAST database of the National Center for Biotechnology Information (NCBI) was used to compare resolved sequences of the 34 isolates with 16S rDNA and rpoB sequences data deposited in GenBank. Alignment of sequences was performed using the Clustal method (ClustalX (1.81)). Analysis of the distance between sequences was made with aid of the phylogenetic analysis using the Fitch program within the Phylogeny Inference Package (PHYLIP).

The NCBI accession numbers for the 16S rRNA gene and rpoB gene sequences of 34 representative isolates determined in this present study are listed in Table 1.

RESULTS

A total of 66 pseudomonads isolates showing fluorescence under UV light, isolated over a period of 18 months from diverse origin, were spread on King's B (KB).

ITS- and BOX-PCR fingerprinting

Figure 1 represents the patterns obtained with ITS-PCR tested with studied *Pseudomonas* fluorescent population (only 32 representative strains are shown). This method applied to 66 isolates relies on polymorphisms within the 16S-23S intergenic spacer regions. A characteristic pattern is obtained that represents the size variations in the multiple rRNA operons. ITS technique showed reproducible patterns consisting of 1 to 4 bands ranging from 300 to 750 bp, but the majority of strains contained only one band. 12 different ITS profiles were produced, encompassing 21 (G1), 1 (G2), 1 (G3), 3 (G4), 1 (G5), 5 (G6), 16 (G7), 1 (G8), 14 (G9), 1 (G10), 1 (G11) and 1 (G12) isolates (Table 1). The discriminatory power of ITS technique was calculated to be 0.797.

Figure 2 represents the pattern obtained with BOX-PCR tested with studied *Pseudomonas* fluorescent population. To gain insight into the genetic diversity and structure of the isolates, BOX-A1R-based repetitive extragenic palindromic-PCR was applied to the 66 isolates. Visual inspection of the DNA fingerprints followed by analysis with Gel-Pro (version 3.1), demonstrated 2 to 12 reproducible bands varying from 100 to 2500 bp with different intensity. 45 distinct profiles were obtained enclosing seven major groups (I to VII) (Table 1). The discriminatory power of Box-PCR method was calculated to be 0.973.

16S rDNA and rpoB sequence analysis and phylogenetic tree

Figures 3 and 4 represent phylogenetic trees obtained with 16S rRNA and rpoB sequences determined in this study for the same strains and the branches with bootstrap value above 70% are shown with spots at nodes.

On the basis of BLAST analysis of 16S rRNA gene (about 700 bp) similarity, species such as *P. aeruginosa*, *Pseudomonas putida*, *Pseudomonas plecoglossicida*, *Pseudomonas monteilii*, *Pseudomonas mosselii*, *P. fluorescens*, *Pseudomonas thivervalensis*, *Pseudomonas otitidis* and *Pseudomonas guezennei* were identified. A total of 19 isolates (PsS2, PsS71, PsS15, PsS28, PsS31, PsS46, PsS48, PsC54, PsS67, PsS75, PsS79, PsWs147, PsTp156, PsC10, PsTp139, PsWs173, PsS83, PsWs140 and PsWt146) belong to *P. putida* / *P. plecoglossicida*, one isolate (PsS11) belongs to *P. monteilii*, one isolate (PsTp171) belongs to *P. mosselii*, six isolates (PsS26, PsS29, PsS73, PsS93, PsS89 and PsWw128) belong to *P. fluorescens* / *P. thivervalensis*, five isolates (PsWw84, PsS150, PsS3, PsC132 and

Table 1. Genetic diversity among pseudomonas strains.

Isolates	16S rDNA accession number	Closest 16S rDNA sequence with type strain	rpoB accession number	Closest rpoB sequence with type strain	ITS profile	BOX profile
PsS150	HM627571	*P. aeruginosa*	JN590039	*P. aeruginosa*	G1	III_2
PsWt157					G1	I_4
PsWw168					G1	III_7
PsWw175					G1	I_2
PsC132	HM627574	*P. aeruginosa*	JN627242	*P. aeruginosa*	G12	III_5
PsWt138					G1	I_3
PsCL10C					G1	I_1
PsTp160					G1	II_2
PsC99					G1	II_1
PsTp179					G1	I_2
PsC5					G1	I_4
PsWw174					G1	III_3
PsS2	HM627611	*P. putida* / *P. plecoglossicida*	JN701899	*P. putida* / *P. syncynea*	G11	III_1
PsS3	HM627570	*P. aeruginosa*	JN620413	*P. aeruginosa*	G1	I_5
PsCLHMC1					G1	III_2
PsWw127					G1	III_3
PsC12					G1	III_3
PsWw84	HM627575	*P. aeruginosa*	JN590033	*P. aeruginosa*	G1	III_6
PsWw121	HM627577	*P. aeruginosa*	JN590036	*P. otitidis*	G1	VI_2
PsTp142					G1	VI_2
PsS89	HM627583	*P. fluorescens* / *P. thivervalensis*	JN590034	*P. moraviensis*	G1	VII_5
PsWw9					G1	VI_2
PsTp139	HM627618	*P. putida* / *P. plecoglossicida*	JN660816	*Pseudomonas* sp.	G2	IV_{13}
PsWs140	HM627619	*P. putida* / *P. plecoglossicida*	JN590037	*P. monteilii*	G3	IV_{13}
PsTp153					G4	IV_{13}
PsTp154					G4	IV_{13}
PsTp155					G4	IV_{13}
PsWw128	HM627582	*P. fluorescens* / *P. thivervalensis*	KC161437	*P. vancouverensis*	G5	VII_4
PsTp169					G6	IV_5
Isolates	**16S rDNA accession number**	**Closest 16S rDNA sequence with type strain**	**rpoB accession number**	**Closest rpoB sequence with type strain**	**ITS profile**	**BOX profile**
PsS11	HM627617	*P. monteilii*	JN634561	*Pseudomonas* sp.	G6	IV_{12}
PsTp172					G6	IV_5
PsS103					G6	IV_8
PsS71	HM627580	*P. putida*/ *P. plecoglossicida*	JN701900	*P. putida* / *P. syncynea*	G7	IV_{11}
PsC10	HM627622	*P. putida* / *P. plecoglossicida*	JN603371	*Pseudomonas* sp.	G7	V_2
PsS73	HM627594	*P. fluorescens* / *P. thivervalensis*	JN704638	*P. aurantiaca* / *P. chlororaphis*	G7	VII_8
PsS26	HM627593	*P. fluorescens* / *P. thivervalensis*	JN704637	*P. aurantiaca* / *P. chlororaphis*	G7	VII_7

Table 1. Contd.

Isolates	16S rDNA accession number	Closest 16S rDNA sequence with type strain	rpoB accession number	Closest rpoB sequence with type strain	ITS profile	BOX profile
PsS29	HM627585	*P. fluorescens* / *P. thivervalensis*	JN660815	*P. aurantiaca* / *P. chlororaphis*	G7	VII$_6$
PsS39					G7	VII$_6$
PsS25					G7	VII$_6$
PsS49					G7	VII$_6$
PsS60					G7	VII$_6$
PsS93	HM627589	*P. fluorescens* / *P. thivervalensis*	JN627241	*P. aurantiaca* / *P. chlororaphis*	G7	VII$_6$
PsS90					G7	VII$_6$
PsS91					G7	VII$_6$
PsS23					G7	VII$_6$
PsWs173	HM627621	*P. putida* / *P. plecoglossicida*	JN711467	*Pseudomonas* sp.	G8	V$_6$
PsS28	HM627596	*P. putida* / *P. plecoglossicida*	JN590028	*P. oleovorans*	G9	IV$_6$
PsS79	HM627629	*P. putida* / *P. plecoglossicida*	JN695065	*P. oleovorans*	G9	IV$_{10}$
PsS48	HM627598	*P. putida* / *P. plecoglossicida*	JN590031	*P. oleovorans*	G9	IV$_9$
PsS102					G9	VII$_2$
PsTp156	HM627607	*P. putida* / *P. plecoglossicida*	JN590040	*P. oleovorans*	G9	IV$_3$
PsWs158					G9	IV$_4$
PsS15	HM627595	*P. putida* / *P. plecoglossicida*	JN634562	*P. oleovorans*	G9	IV$_1$
PsC54	HM627597	*P. putida* / *P. plecoglossicida*	JN603372	*P. oleovorans*	G10	IV$_7$
PsS4	HM627612	*Pseudomonas* sp.	JN590027	*P. plecoglossicida*	G9	III$_8$
PsS46	HM627613	*P. putida* / *P. plecoglossicida*	JN590030	*P. oleovorans*	G6	IV$_1$
PsS31	HM627624	*P. putida* / *P. plecoglossicida*	JN590039	*P. oleovorans*	G9	VII$_1$
PsS67	HM627602	*P. putida* / *P. plecoglossicida*	JN695063	*P. oleovorans*	G9	VII$_3$
PsS18					G9	VII$_3$
PsWs147	HM627623	*P. putida* / *P. plecoglossicida*	JN620414	*P. oleovorans*	G9	V$_5$
PsS75	HM627609	*P. putida* / *P. plecoglossicida*	JN695064	*P. oleovorans*	G9	V$_3$
PsS83	HM627581	*P. putida* / *P. plecoglossicida*	JN590032	*P. corrugata*	G7	VI$_1$
PsWt146	HM627604	*P. putida* / *P. plecoglossicida*	JN590038	*P. monteilii*	G7	V$_4$
PsTp171	HM627603	*P. mosselii*	JN590041	*P. mosselii*	G7	V$_1$
PsWw118	HM627606	*P. otitidis* / *P. guezennei*	JN590035	*P. otitidis*	G1	VI$_3$

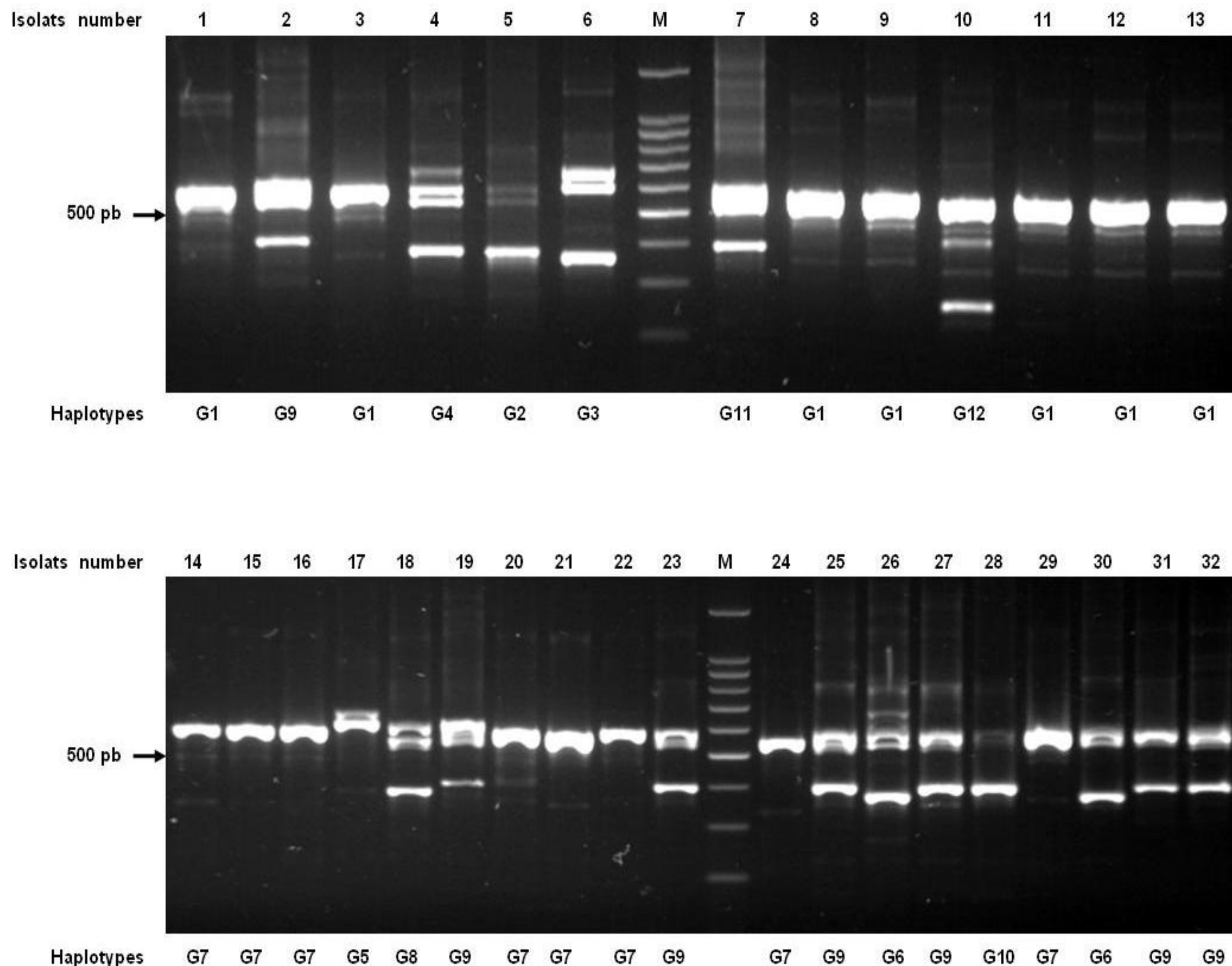

Figure 1. ITS profiles of *Pseudomonas* fluorescent isolates. M: 100 pb molecular size marker. Lanes 1- PsWw.118 (G1), 2- PsS.31 (G9), 3- PsWw.121 (G1), 4- PsTp.153 (G4), 5- PsTp.139 (G2), 6- PsWs.140 (G3), 7- PsS.2 (G11), 8- PsS.3 (G1), 9- PsS.89 (G1), 10- PsC.132 (G12), 11- PsTp.179 (G1), 12- PsWw.127 (G1), 13-PsS.150 (G1), 14- PsS.73 (G7), 15-PsS.26 (G7), 16- PsS.93 (G7), 17- PsWw.128 (G5), 18- PsWs173 (G8), 19- PsWs.147 (G9), 20- PsTp.171 (G7), 21- PsWt.146 (G7), 22- PsS.83 (G7), 23- PsS.75 (G9), 24- PsC.10 (G7), 25- PsS.4 (G9), 26- PsS.11 (G6), 27- PsS.15 (G9), 28- PsC.54 (G10), 29- PsS.71 (G7), 30- PsS.46 (G6), 31- PsS.79 (G9) and 32- PsS.28 (G9).

PsWw121) belong to *P. aeruginosa*, one isolate (PsWw118) belongs to *P. otitidis* / *P. guezennei*, and one isolate (PsS4) belongs to *Pseudomonas* sp. Phylogenetic analyses of 34 representative fluorescent pseudomonad isolates based on neighbour-joining method with 1000 bootstrap sampling resulted in three major clusters (Table 1 and Figure 3). Of the 34 isolates, cluster I was formed by 22 isolates, representing the major group, cluster II was formed by 6 strains and cluster III was formed by 6 strains.

On the basis of phylogenetic analysis of *rpoB* gene (about 1000 bp) similarity, species such as *P. aeruginosa*, *P. putida*, *P. plecoglossicida*, *P. monteilii*, *P. mosselii*, *Pseudomonas syncynea*, *Pseudomonas corrugata*, *Pseudomonas oleovorans*, *Pseudomonas aurantiaca*, *Pseudomonas chlororaphis*, *Pseudomonas moraviensis*, *P. otitidis* and *Pseudomonas vancouverensis* were identified. A total of four isolates (PsS3, PsC132, PsWw84 and PsS150) belong to *P. aeruginosa*, two isolates (PsWw118 and PsWw121) belong to *P. otitidis*, one isolate (Ps4) belongs to *P. plecoglossicida*, 11 isolates (PsS15, PsS28, PsS79, PsTp156, PsS31, PsS46, PsS48, PsC54, PsS67, PsS75 and PsWs147) belong *P. oleovorans*, one isolate (PsS83) belongs to *P. corrugata*, two isolates (PsWt146 and PsWs140) belong to *P. monteilii*, two isolates (PsS2 and PsS71) belong to *P. putida* / *P. syncynea*, one isolate (PsTp171) belongs to *P. mosselii*, four isolates (PsS26, PsS29, PsS73 and PsS93) belong to *P. aurantiaca* / *P. chlororaphis*, one isolate (PsS89) belongs to *P.*

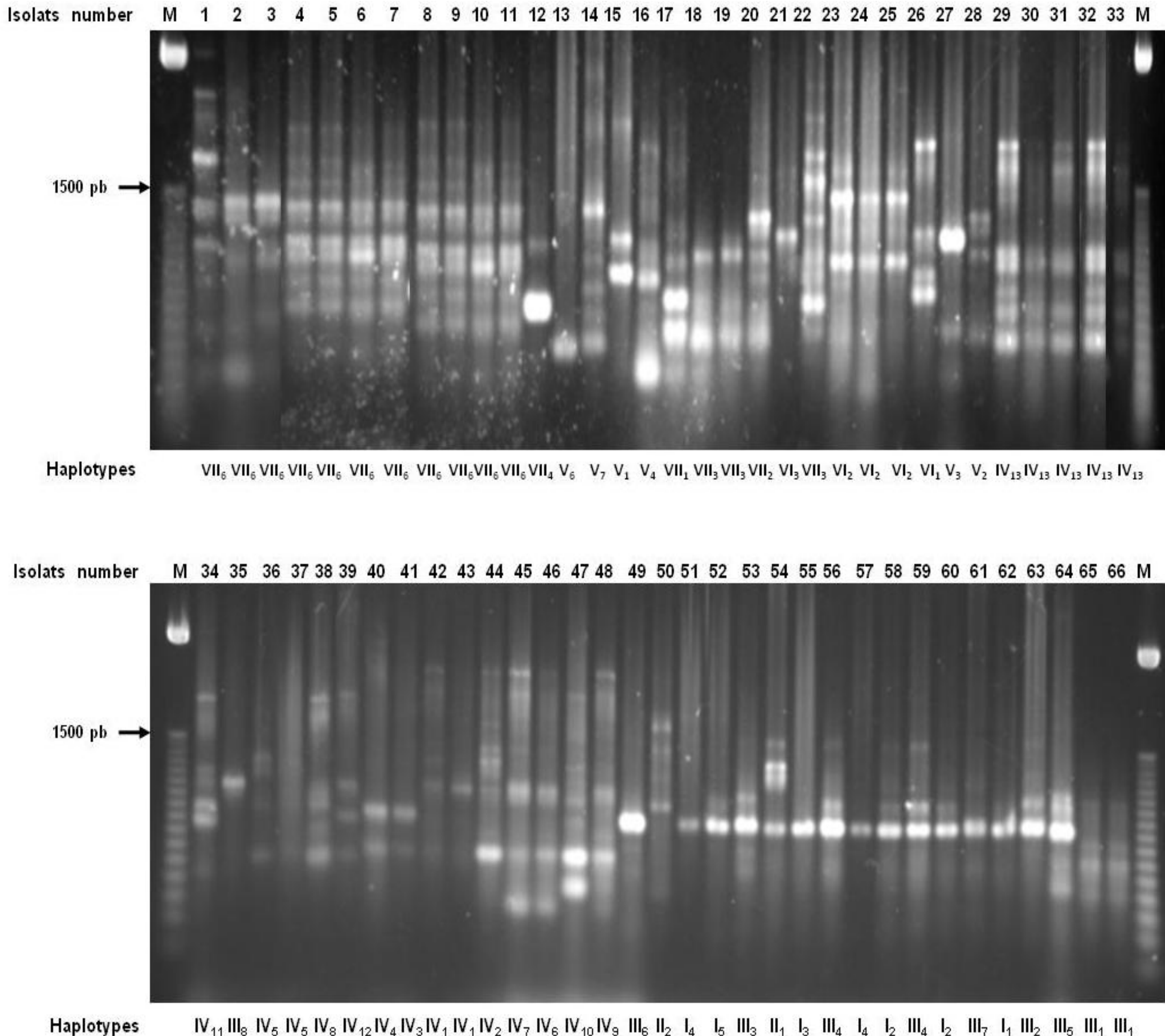

Figure 2. BOX profiles of *Pseudomonas* fluorescent isolates. M: 50 pb molecular size marker. Lanes 1- PsS.73 (VII_6), 2- PsS.26 (VII_6), 3- PsS.60 (VII_6), 4- PsS.49 (VII_6), 5- PsS.23 (VII_6), 6- PsS.90 (VII_6), 7- PsS.91 (VII_6), 8- PsS.93 (VII_6), 9- PsS.29 (VII_6), 10- PsS.25 (VII_6), 11- PsS.39 (VII_6), 12- PsWw.128 (VII_4), 13- PsWs.173 (V_6), 14- PsWs.147 (V_7), 15-PsTp.171 (V_1), 16- PsWt.146 (V_4), 17-PsS.31(VII_1), 18- PsS.18 (VII_3), 19- PsS.67 (VII_3), 20- PsS.102 (VII_2), 21- PsWw.118 (VI_3), 22- PsS.89 (VII_5), 23- PsTp.142 (VI_2), 24- PsWw.121 (VI_2), 25- PsWw.9 (VI_2), 26- PsS.83 (VI_1), 27- PsS.75 (V_3), 28- PsC.10 (V_2), 29- PsTp.155 (IV_{13}), 30- PsTp.154 (IV_{13}), 31- PsTp.153 (IV_{13}), 32- PsTp.140 (IV_{13}), 33- PsTp.139 (PsTp.155 (IV_{13}), 34- PsS.71(IV_{11}), 35- PsS.4(III_8), 36- PsTp.172(IV_5), 37- PsTp.169(IV_5), 38- PsS.103(IV_8), 39- PsS.11(IV_{12}), 40- PsWs.158 (IV_4), 41- PsTp.156 (IV_3), 42- PsS.15 (IV_1), 43- PsS.46 (IV_1), 44- PsWw.124 (IV_2), 45- PsC.54 (IV_7), 46- PsS.28 (IV_6), 47- PsS.79 (IV_{10}), 48- PsS.48 (IV_9), 49- PsWw.84 (III_6), 50- PsTp.160(II_2), 51- PsS.3 (I_4), 52- PsC.5 (I_5), 53- PsC.12 (III_3), 54- PsC.99 (II_1), 55- PsWt.138 (I_3), 56- PsWw.174 (III_4), 57- PsWt.157 (I_4), 58- PsTp.179 (I_2), 59- PsWw.127 (III_4), 60- PsWw.175 (I_2), 61- PsWw168 (III_7), 62- PsCL.10C (I_1), 63- PsS.150 (III_2), 64- PsC.132 (III_5), 65- PsS.2 (III_1) and 66- PsCL.HMC1 (III_1).

moraviensis, one isolate (PsWw128) belongs to *P. vancouverensis* and four isolates (PsC10, PsS11, PsWs173 and Ps139) belong to *Pseudomonas* sp. Phylogenetic analyses of 34 representative fluorescent pseudomonad isolates based on NJ method with 1000 bootstrap sampling resulted into three six clusters (Table 1 and Figure 4). Of the 34 isolates, cluster I contained 20 isolates, representing the major group, cluster II contained six strains, cluster III contained one strain, cluster IV contained four strains, cluster V contained two

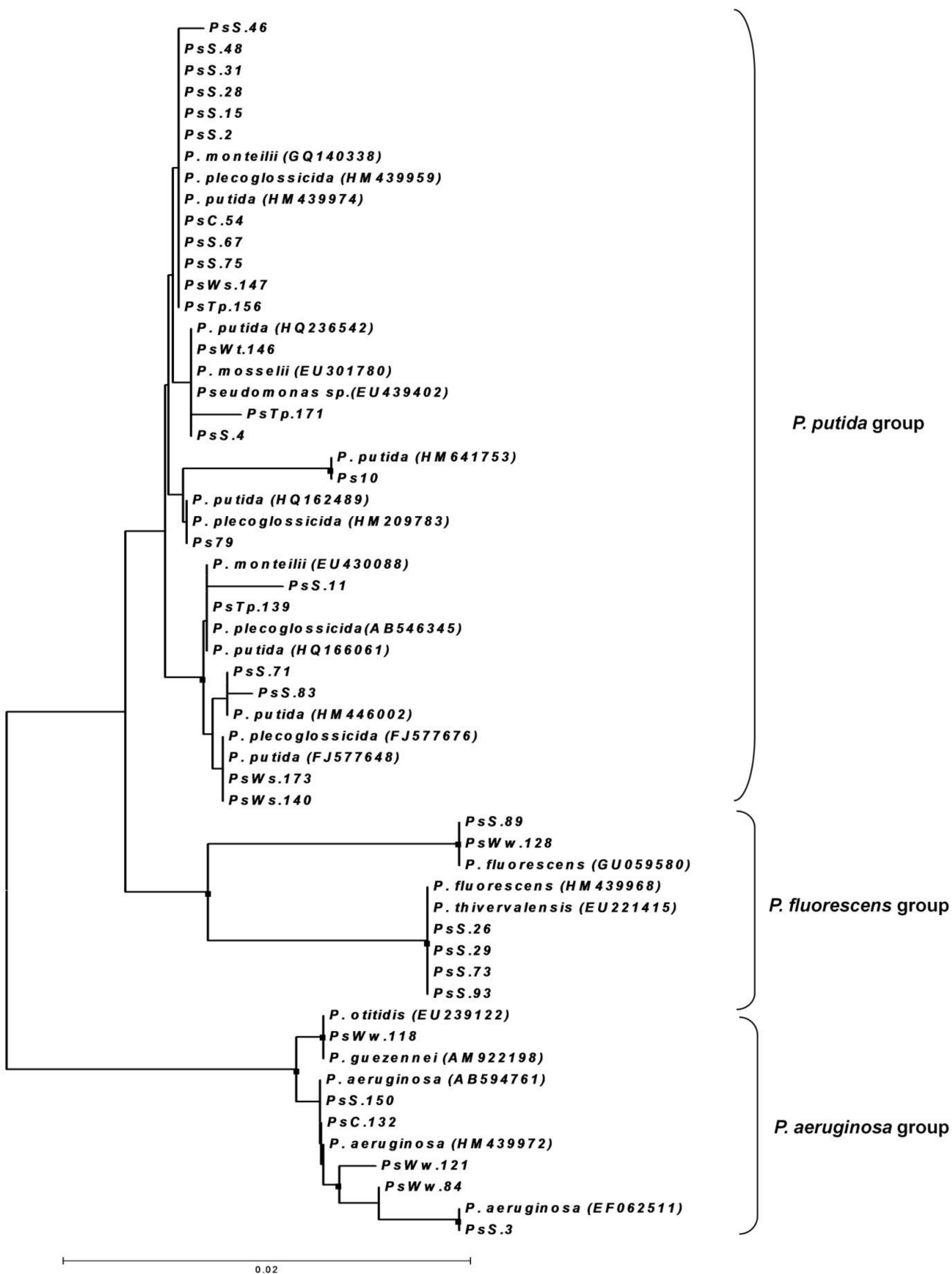

Figure 3. Neighbor-joining phylogenetic tree of 16S rRNA sequences of 34 isolates and their closest phylogenetic relatives. Sequences of the compared strains were obtained from databases, and the accession numbers are in parenthesis. The tree topology was constructed using ClustalX (1.81).

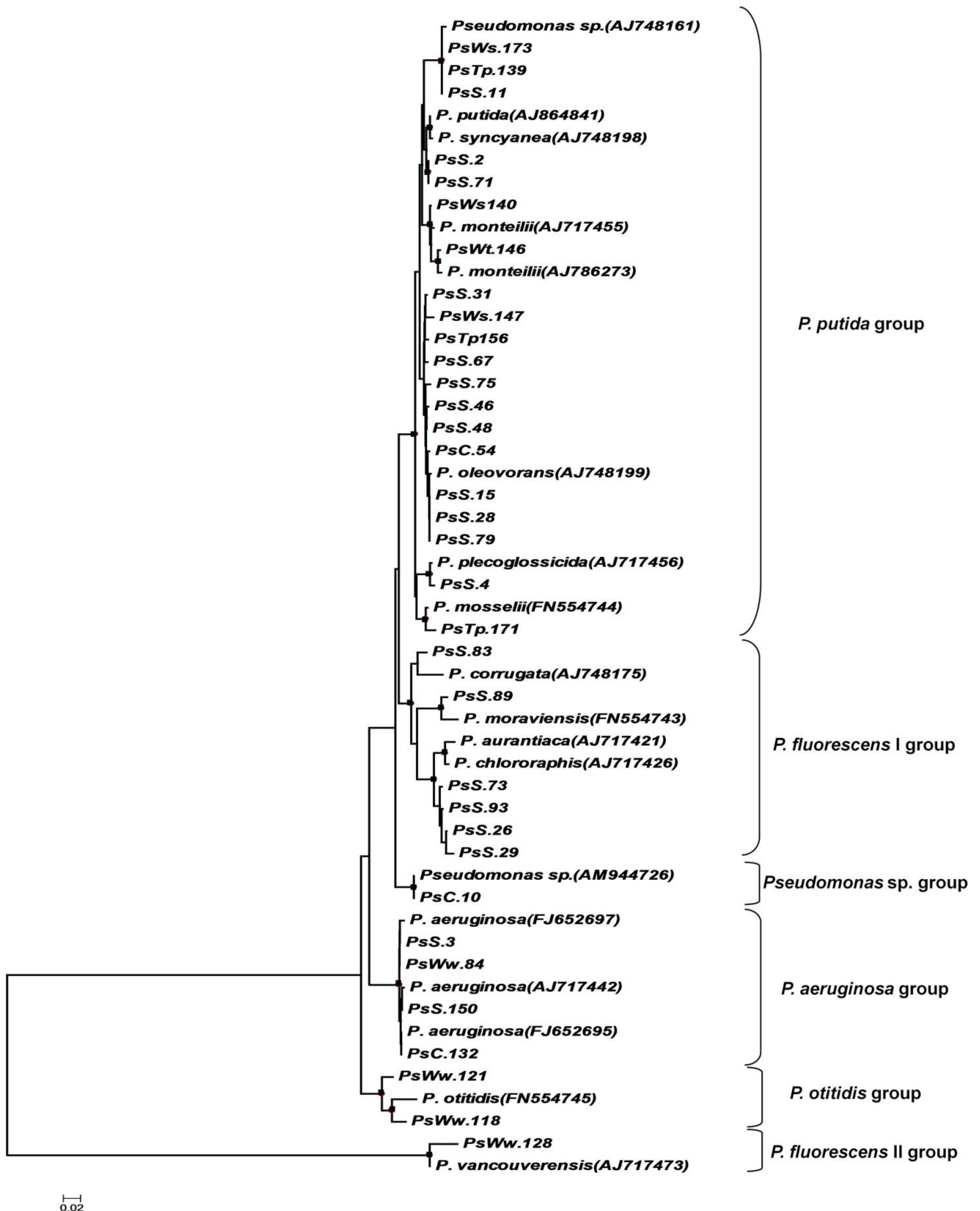

Figure 4. Neighbor-joining phylogenetic tree of *RpoB* sequences of 34 isolates and their closest phylogenetic relatives. Sequences of the compared strains were obtained from databases, and the accession numbers are in parenthesis. The tree topology was constructed using ClustalX (1.81).

strains and cluster VI contained one strain.

DISCUSSION

The involvement of some fluorescent *Pseudomonas* species in plant diseases and some others as powerful plant growth-promoting rhizobacteria, together with the demonstration of pyoverdines as biocontrol agents implicated in such processes (Meyer, 2000), shows that an improved classification must be developed.

In this investigation, BOX-PCR, using a single primer, showed a higher discriminatory power and polymorphism than ITS-PCR. Jensen et al. (1993) reported that ITS polymorphisms may form the PCR based identification of many bacterial species, as well as differentiating between certain bacteria at subspecies level. Our results differed from that in this findings, in that, the ITS-PCR typing method did not seem to be a very logical division of species when compared with the other methods. For example, PsWw.118 (identified as *P. otitidis*) and PsS.150 (identified as *P. aeruginosa*) are grouped into the same haplotype (G1). This was largely due to the limited number of bands in each group, which did not provide sufficient characters for confident differentiation of all genomovars (Dawson et al., 2002). In order to increase the number of comparable characters, other authors have found that ITS-PCR typing can be improved by the digestion of the PCR products with the restriction endonucleases (Natalini and Scortichini, 2007; Scarpellini et al., 2004).

In recent study of Popavath et al. (2008), the BOX-PCR genomic fingerprints produced 62 BOX profiles within a total of 95 isolates of fluorescent pseudomonads isolated from banana root-adhering soil. In fact BOX-PCR genomic fingerprint was able to differentiate strains very closely related. This method allowed the recognition of 14 different profiles among the *P. aeruginosa* isolates, which reflected the high degree of interspecies diversity (popavath et al., 2008). Furthermore, a common band was observed (400 bp) in all *P. aeruginosa* isolates patterns which could allow the recognition of this specie. BOX-PCR may be better as it is targeted to a specific region of the entire genome, including the variable regions that do not undergo a large pressure selection, while ribosomal operons are highly conserved (Versalovic et al., 1991). Another study conducted by Shanmugam et al. (2008) reinstated the importance of whole-cell protein analysis in assessing the diversity of pseudomonads. Olive and Bean (1999) showed that BOX-PCR have a better strain differentiation power, easier to perform, quicker and cheaper than ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP) and other techniques.

Based on phylogenetic characterization, only 9 branches in the 16S rRNA tree are supported by bootstrap values above 70% while the *rpoB* tree contains much more branches (17 branches). Furthermore, comparison of the 16S rRNA and *rpoB* phylogenetic trees led us to observe generally longer branches in the *rpoB* tree than in the 16S rRNA tree (while considering the scale at 0.02). In addition, the sequencing of the *rpoB* gene has identified 13 different species and sub-species while the 16 rRNA gene sequencing differentiated only 9 species. Obviously, higher phylogenetic resolution was obtained with *rpoB* sequences. This result is in agreement with the findings of Ait Tayeb et al. (2005) that showed approximately three-times-higher taxonomic resolution of the *rpoB* tree than that of 16S rRNA tree. However, functional genes such as *rpoB* could be used to target a more precise phylogeny and for a higher taxonomic resolution, because of its essential role in cellular metabolism (Ait Tayeb et al., 2005).

Conclusions

Results of this investigation have demonstrated that BOX-PCR show a good discriminative ability with reproducibility, stability, low cost and can be used as a rapid method of comparing fairly homogenous pseudomonads strains for genetic diversity. In addition, the *rpoB* sequencing seems to be most suitable for identification of *Pseudomonas* strains at species level. The three-times-higher taxonomic resolution will facilitate assignation of a strain to a species. The *rpoB* sequencing could significantly extend *Pseudomonas* phylogeny to provide better picture of *Pseudomonas* diversity.

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