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ORIGINAL ARTICLE

Development of Genomic Tools for the Identification of Certain *Pseudomonas* up to Species Level

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Abstract *Pseudomonas* is a highly versatile bacterium at the species level with great ecological significance. These genetically and metabolically diverse species have undergone repeated taxonomic revisions. We propose a strategy to identify *Pseudomonas* up to species level, based on the unique features of their 16S rDNA (*rrs*) gene sequence, such as the frame work of sequences, sequence motifs and restriction endonuclease (RE) digestion patterns. A species specific phylogenetic framework composed of 31 different *rrs* sequences, allowed us to segregate 1,367 out of 2,985 *rrs* sequences of this genus, which have been classified at present only up to genus (*Pseudomonas*) level, as follows: *P. aeruginosa* (219 sequences), *P. fluorescens* (463 sequences), *P. putida* (347 sequences), *P. stutzeri* (197 sequences), and *P. syringae* (141 sequences). These segregations were validated by unique 30–50 nucleotide long motifs and RE digestion patterns in their *rrs*. A single gene thus provides multiple makers for identification and surveillance of *Pseudomonas*.

Keywords *Pseudomonas* · Diversity · Evolution · Framework · Phylogeny · Restriction endonuclease

Introduction

Gene and genome sequencing has tremendously increased our knowledge of the microbes. The most extensively studied among these is 16S rDNA (*rrs*) gene [1] (http://rdp.cme.msu.edu). Most molecular techniques allow identification of organism up to genus level [2]. An alternative to full length *rrs* sequence is the multi-locus sequence analysis or multi locus sequence typing, which cannot correctly interpret phylogenetic differences among closely related species within *Acinetobacter* and *Pseudomonas* [2–4]. *Pseudomonas* is a genetically and metabolic diverse genus that has undergone repeated taxonomic revisions [5, 6]. Phylogenetic analysis based on concatenated sequences of four core HKGs (housekeeping genes—*rrs*, *gyrB*, *rpoB* and *rpoD*), allowed segregation of different groups as (i) intrageneric cluster I composed of *P. aeruginosa*, *P. flavescens*, *P. mendocina*, *P. resinovorans*, [3] and (ii) *P. fluorescens* intrageneric cluster II consisting of *P. chlororaphis*, *P. fluorescens*, *P. syringae* and *P. putida* [7] Studies based on the nucleotide sequences of the genes *rrs*, *gyrB* and *rpoD* [8–10], revealed that *P. aeruginosa* and *P. stutzeri* form a group quite distinct from that constituted by *P. syringae*, *P. fluorescens* and *P. putida*. In spite of these extensive analyses, a definitive lineage could not be established and hence needs re-evaluation [3, 11]. Recent works on exploring the latent features of *rrs* have revealed reliable framework sequences, unique motifs and *in silico* restriction mapping, allowing species level identification of organisms in the cases of *Bacillus*, *Clostridium*, *Stenotrophomonas* and *Streptococcus* [6, 12–14]. The genomic tools developed in

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these studies can be extended to discriminate novel taxa and may prove helpful to classify an organism, when it encounters a never-seen-before situation [15]. In this work, we have employed around 1,350 *rrs* gene sequences (each >1,200 nucleotides, nts) of five species of *Pseudomonas* sensu stricto to select unique molecular markers within *rrs* sequences, to be used as phylogenetic frameworks for identifying *Pseudomonas* up to species level. The results were validated through the presence of molecular markers: unique motif(s) and restriction endonuclease (RE) digestion patterns.

Materials and Methods

Pseudomonas rrs sequence were retrieved from RDP/NCBI sites: http://rdp.cme.msu.edu/; http://www.ncbi.nlm.nih.gov/ (Supplementary Tables 1, 2). The phylogenetic, restriction enzyme sites and *Pseudomonas* species-specific motif analysis among of *rrs* sequences has been detailed as a Supplementary Material 2.

Results

Phylogenetic Frame Work for *Pseudomonas* Species

On the basis of 1,350 *rrs* sequences (>1,200 nts each) belonging to group I *Pseudomonas* spp.: *P. aeruginosa* (375 sequences), *P. fluorescens* (273 sequences), *P. putida* (414 sequences), *P. stutzeri* (199 sequences), and *P. syringae* (89 sequences) (Supplementary Tables 1, 2), phylogenetic frame was developed. It was employed to identify 2,985 *rrs* sequences belonging to organisms designated at present only up to genus level (Supplementary Table 2). For *P. aeruginosa*, four phylogenetic trees based on 375 *rrs* sequences enabled us to select eight frame work sequences (FWS) (Supplementary Table 3; Supplementary Fig. 1). Similarly, we could select seven FWS each of *P. fluorescens* and *P. putida*, five FWS in the case of *P. stutzeri* and four FWS for *P. syringae* (Supplementary Table 3; Supplementary Figs. 1–3). The total genetic diversity of 1,350 *rrs* sequences belonging to five *Pseudomonas* spp. could be represented by 31 as FWS including 15 type strains. A phylogenetic tree based on these 31 FWS (Fig. 1; Table 1) showed high heterogeneity (low Bootstrap values) within each *Pseudomonas* species (Figs. 1, 2, 3), enabling easy separation into distinct groups.

Validation of Species-Specific Phylogenetic FWS

Phylogenetic trees (29) of *rrs* sequences each belonging to *Pseudomonas* sp. along with species specific phylogenetic framework composed of 31 *rrs* sequences, revealed that 1,367 *rrs* sequences belonged to *P. aeruginosa* (219 sequences), *P. fluorescens* (463 sequences), *P. putida* (347 sequences), *P. stutzeri* (197 sequences), and *P. syringae* (141 sequences) (Supplementary Table 4). Final trees to demonstrate that these *Pseudomonas* spp. fall well within their respective *Pseudomonas* groups., a small subset was selected from each species as follows: 66 sequences for *P. aeruginosa* (Fig. 2; Supplementary Table 4), 129 sequences for *P. fluorescens* (Figs. 3, 4), 75 sequences for *P. putida* (Fig. 5; Supplementary Table 4), 66 sequences for *P. stutzeri* (Supplementary Fig. 4; Supplementary Table 4), and 65 sequences *P. syringae* (Supplementary Fig. 5; Supplementary Table 4). At this rate about 42.41 % of the presently unclassified *Pseudomonas* sp. could be identified up to species level.

In silico RE Digestion Patterns

In the present study, six REs viz *Alu*I, *Bfa*I, *Dpn*II, *Hae*III, *Rsa*I and *Tru*9I were found to be effective in drawing meaningful conclusions (Table 2; Supplementary Table 5).

AluI

In silico digestion of *rrs* sequences of five *Pseudomonas* spp. (Table 2) with *Alu*I resulted in digestion patterns, which could distinguish them into three major groups. Group 1 with a *Alu*I pattern of 162-403-211-207-209-162 nts fragments occurred among *P. aeruginosa* (321/375 sequences), *P. putida* (254/414 sequences) and *P. stutzeri* (143/199 sequences). Since, these three *Pseudomonas* spp. were indistinguishable, we may propose them to have a common ancestor. Group 2 was composed of *rrs* sequences belonging to *P. fluorescens*. Here two different genetic events seem to have happened simultaneously: (1) shift in the *Alu*I site between the fragments 403 and 211 nts, leading to the appearance of two new fragments of 559 and 55 nts, and (2) appearance of an additional RE site in the 211 nts fragment leading to two fragments of 196 and 15 nts. Further evolution among *P. fluorescens* seems to have happened at 5′ end in 55/273 sequences such that a distinct 162 nts fragment is no longer evident. Another subpopulation of *P. fluorescens* (30/273 sequences) seems to have evolved by the disappearance of *Alu*I sites as 5′ and 3′ ends. Organisms belonging to *P. syringae* had a *Alu*I digestion pattern of 403-196-15-207-209-162 nts in their *rrs* at a frequency of 79/89 sequences. This pattern in *P. syringae* was indistinguishable from that observed in a small population (55/273 sequences) of *P. fluorescens*. In conclusion, we may state that *P. aeruginosa*, *P. putida* and *P. stutzeri* cannot be distinguished among themselves on the basis of *Alu*I digestion of their *rrs*. *P. fluorescens* has

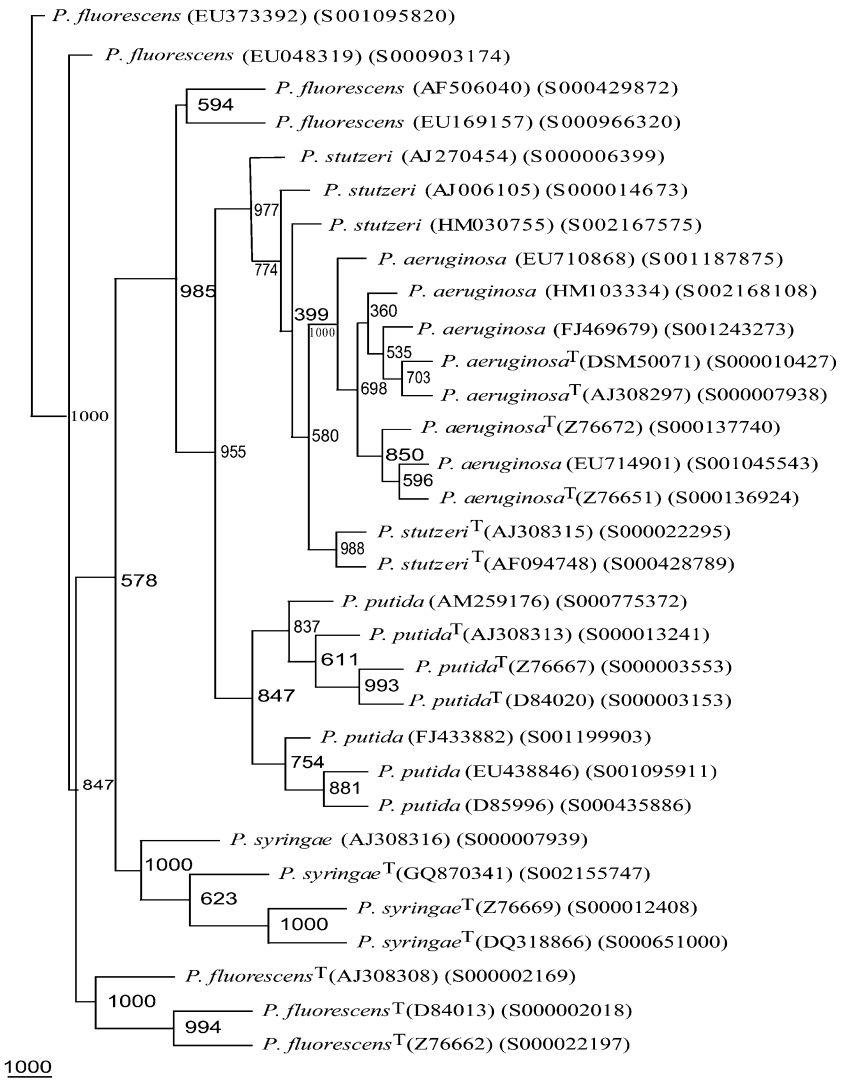

Fig. 1 Phylogenetic tree of 31 *rrs* framework sequences of *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. stutzeri*, and *P. syringae*. *T* type strain

Table 1 Accession numbers of *rrs* sequences of *Pseudomonas* species used for generating phylogenetic framework (http://www.ncbi.nlm.nih.gov/ and http://rdp.cme.msu.edu/)

Organism	Reference sequence(s)
P. aeruginosa	EU714901, FJ469679, EU710868, HM103334, X06684(T), AJ308297(T), Z76651(T), Z76672(T)
P. fluorescens	AF506040, EU169157, EU048319, EU373392, D84013(T), AJ308308(T), Z76662(T)
P. putida	D85996, AM259176, EU438846, FJ433882, D84020(T), Z76667(T), AJ308313(T)
P. stutzeri	AJ270454, AJ006105, HM030755, AJ308315(T), AF094748(T)
P. syringae	GQ870341, AJ308316(T), Z76669(T), DQ318866(T)
Total	31 strains

Type strain designated as (T)

evolved into different populations and that *P. syringae* resembles one of the subgroups of *P. fluorescens*.

DpnII

*Dpn*II digestion patterns of *rrs* among *Pseudomonas* spp. (Table 2), lead to three groups. *P. fluorescens* and *P. putida* were indistinguishable due to a similar RE pattern: 24-83-906-12 nts. *P. aeruginosa* (200/375 sequences) and *P. stutzeri* (118/199 sequences) had similar *Dpn*II pattern in their *rrs*: 77-24-83-449-9-448-12. Among the five *Pseudomonas* spp., *P. syringae* had a unique *Dpn*II pattern: 262-24-83-906-12-225, intermediate to the previous two groups. Here certain RE sites seem to have disappeared resulting in the merger of small fragments such as 185-77 into 262 nts and 449-9-448 into a single fragment and

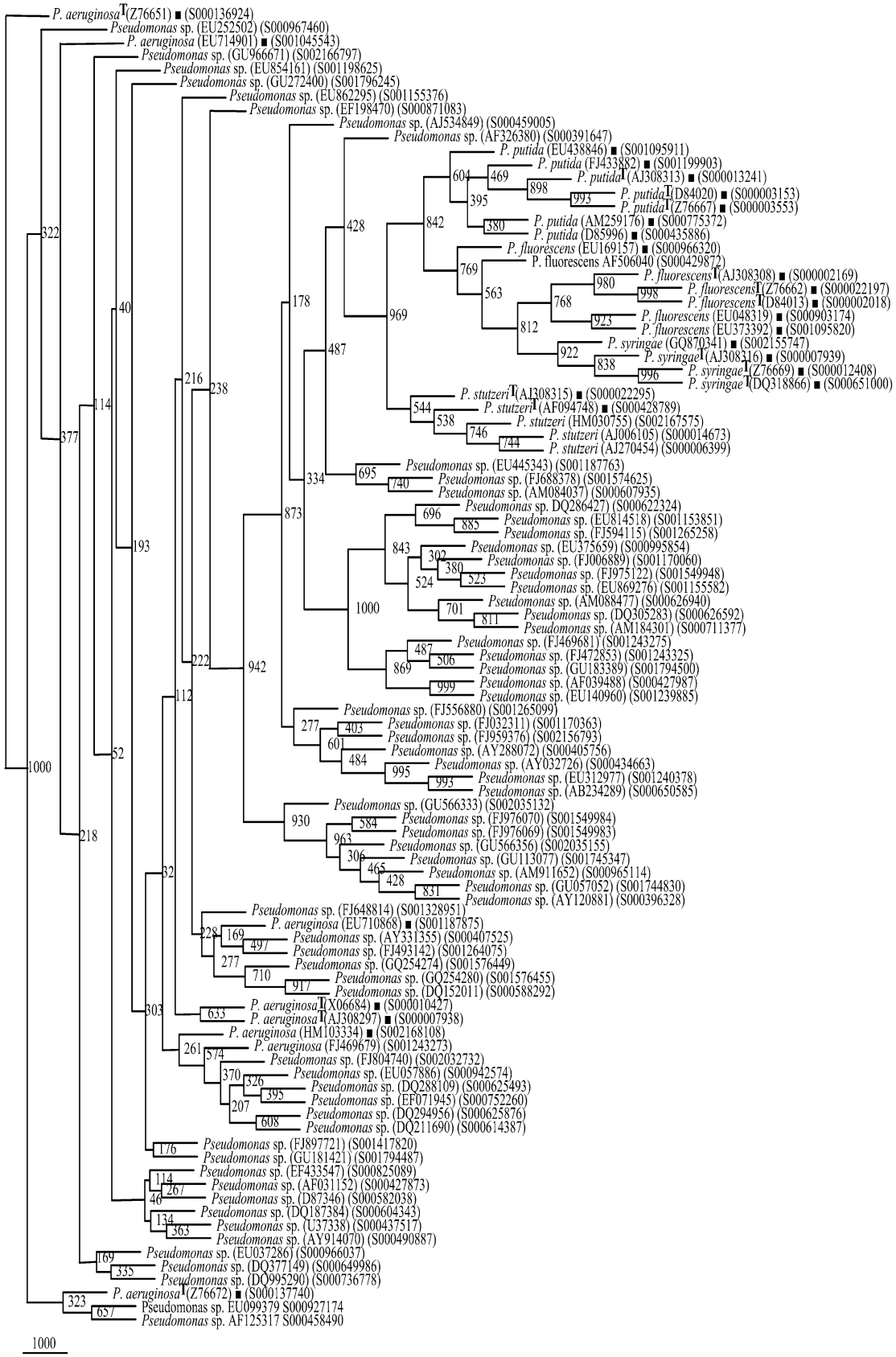

Fig. 2 Phylogenetic tree of *rrs* of *Pseudomonas* sp. (66 segregated as *P. aeruginosa*, Supplementary Table 4) and Framework sequences (*Filled squares*) (Fig. 1)

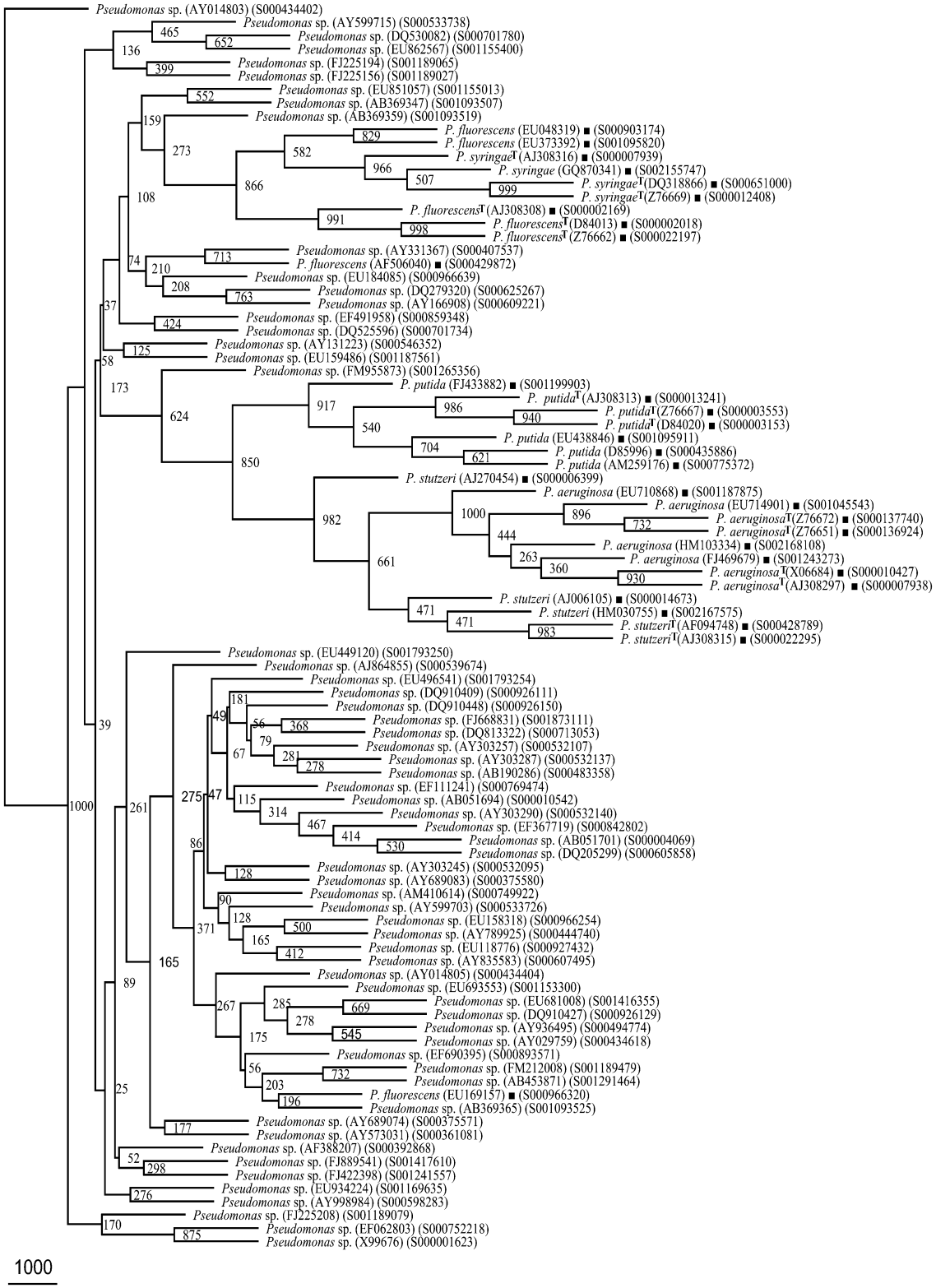

Fig. 3 Phylogenetic tree of *rrs* of *Pseudomonas* sp. (61 segregated as *P. fluorescens*, Supplementary Table 4) and Framework sequences (Fig. 1). 61/129 have been presented here. The rest 68 *rrs* sequences have been presented in Fig. 4

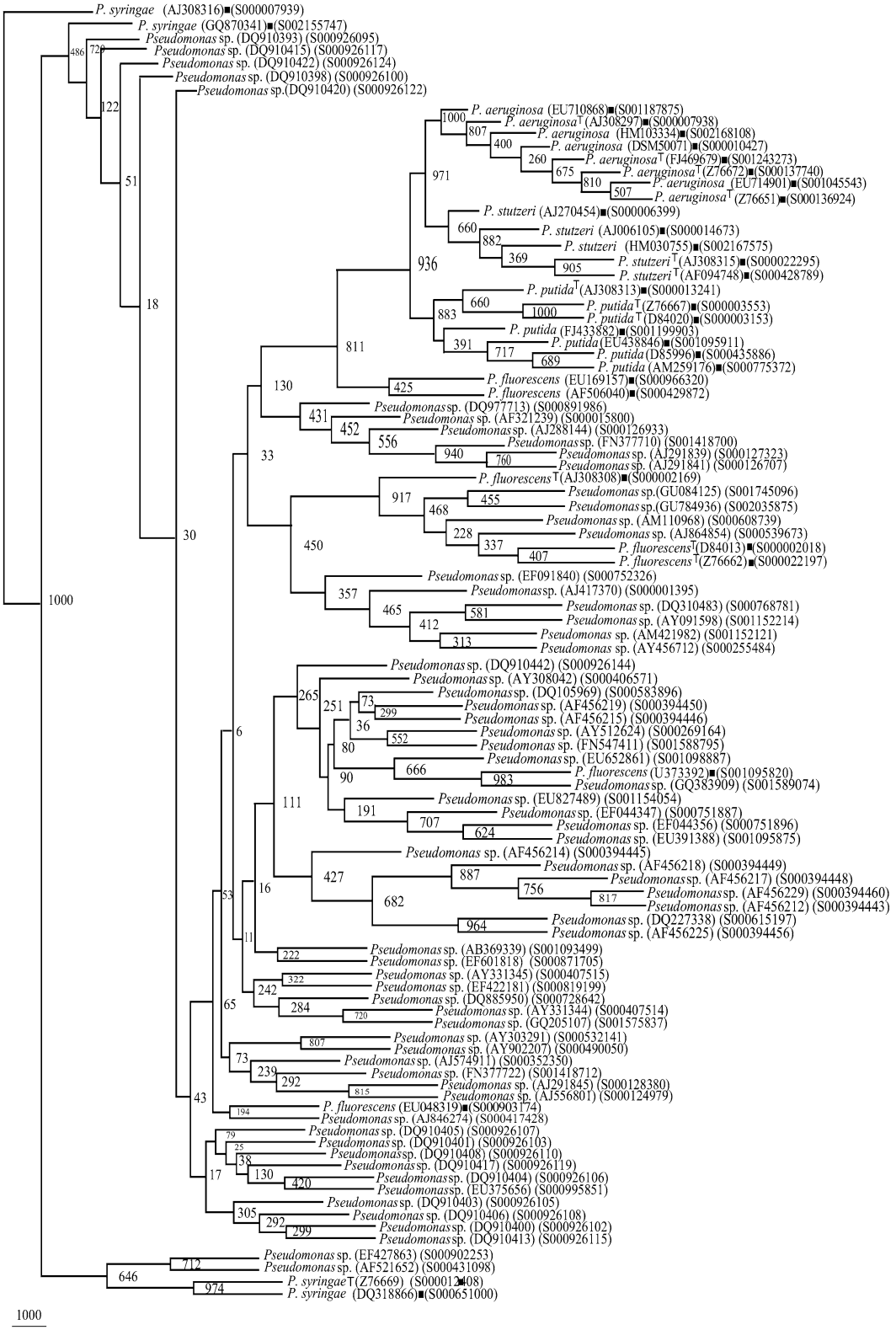

Fig. 4 Phylogenetic tree of *rrs* of *Pseudomonas* sp. (68 segregated as *P. fluorescens*, Supplementary Table 4) and Framework sequences (Fig. 1)

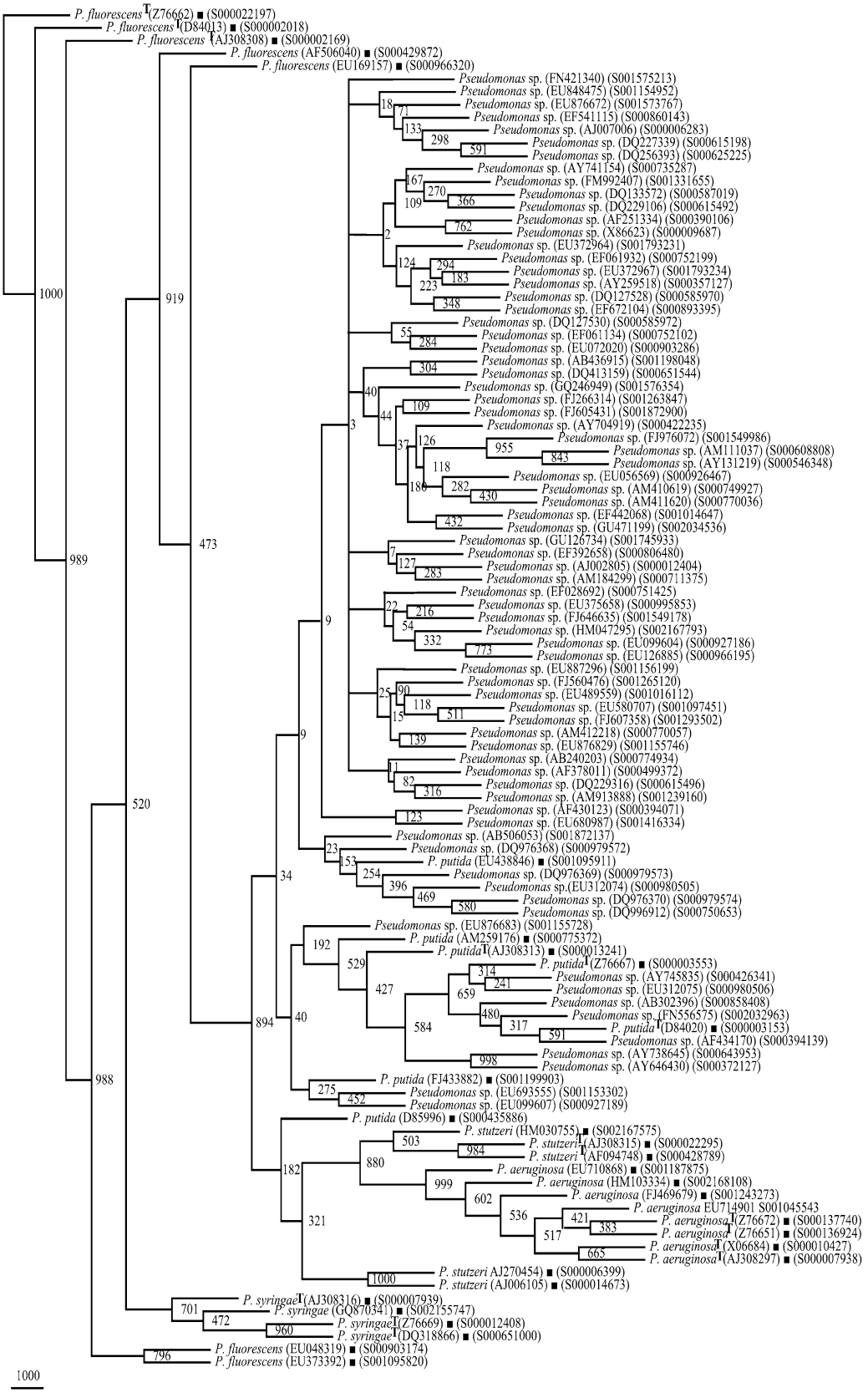

Fig. 5 Phylogenetic tree of *rrs* of *Pseudomonas* sp. (75 segregated as *P. putida*, Supplementary Table 4) and Framework sequences

Table 2 *In silico* RE activity in *rrs* of *Pseudomonas* spp.: *Alu*I and *Dpn*II

Pseudomonas spp.	F[a]	RE digestion fragments (nucleotides, nts)																			
		*Alu*I																			
P. putida	254/414	*	162	*		403		*		211		*		207		*	209	*	162	*	
P. putida	54/414	*	162	*		403		*	195	*	15	*		207		*	209	*	162	*	
P. putida	61/414	*	162	*	215	*	188	*		211		*		207		*	209	*	163	*	
P. aeruginosa	321/375	*	162	*		403		*		211		*		206		*	208	*	162	*	
P. aeruginosa	19/375	*	158	*		403		*				418				*	208	*	162	*	
P. stutzeri	143/199	*	162	*		403		*		211		*		207		*	209	*	162	*	
P. stutzeri	13/199	*	162	*	137	*	264	*		211		*		207		*	208	*	162	*	
P. stutzeri	16/199	*	162	*		402		*		211		*	175	*	32	*	209	*	162	*	
P. syringae	79/89			*		403		*	196	*	15	*		207		*	209	*	162	*	
P. fluorescens	116/273	*	160	*			599			*	15	*		207		*	209	*	162	*	
P. fluorescens	47/273	*	162	*		402		*	196	*	15	*		207		*	209	*	162	*	
P. fluorescens	55/273			*		403		*	196	*	15	*		207		*	209	*	162	*	
P. fluorescens	30/273			*		403		*	196	*	15	*		207		*	209	*			
		*Dpn*II																			
P. aeruginosa	123/375	*	183	*	77	*	24	*	83	*	449	*	9	*	447	*	12	*		225		*
P. aeruginosa	200/375			*	77	*	24	*	83	*	449	*	9	*	447	*	12	*				
P. aeruginosa	13/375			*	77	*	24	*	83	*	449	*	9	*	447	*	12	*	170	*		
P. stutzeri	29/199	*		260		*	24	*	83	*	449	*	9	*	448	*	12	*		225		*
P. stutzeri	118/199			*	77	*	24	*	83	*	449	*	9	*	448	*	12	*				
P. stutzeri	31/199	*	183	*	77	*	24	*	83	*	449	*	9	*	448	*	12	*		225		*
P. syringae	88/89	*		262		*	24	*	83	*			906			*	12	*		225		*
P. fluorescens	148/273					*	24	*	83	*			906			*	12	*				
P. fluorescens	23/273					*	24	*	83	*			906			*	12	*		225		*
P. fluorescens	20/273			*	77	*	24	*	83	*			906			*	12	*				
P. fluorescens	51/273	*		260		*	24	*	83	*			906			*	12	*		225		*
P. putida	250/414					*	24	*	83	*			906			*	12	*				
P. putida	17/414					*	24	*	83	*			906			*	12	*		225		*
P. putida	103/414	*		260		*	24	*	83	*			906			*	12	*				
P. putida	31/414	*		260		*	24	*	83	*			905			*	12	*	207		*	

[a] Frequency of organisms showing this RE digestion pattern. Asterisk indicates RE site in the *rrs* sequences

906 nts. Incidentally, this unique pattern of *P. syringae* (88/89 *rrs* sequences) matches with a small population of *P. fluorescens* (51/273 *rrs* sequences). Each of the *Pseudomonas* spp., except *P. syringae* showed subgroups, where they show resemblance to those from other subgroups. Unlike *Alu*I digestion pattern of *rrs* of *Pseudomonas* spp., where *P. aeruginosa*, *P. putida* and *P. stutzeri* were indistinguishable, here, with *Dpn*II, *P. putida* could be separated out of this group. The observations made with *Dpn*II, once again supported that *P. syringae* is likely to have evolved as a sub population of *P. fluorescens* as was also observed with *Alu*I.

The information on the digestion patterns generated by REs—*Hae*III, *Rsa*I, *Tru*9I and *Bfa*I have been presented as Supplementary Material 1 and Tables (Supplementary Table 5).

In brief, though not very effective as a tool to distinguish *Pseudomonas* spp. with authenticity, it does provide an opportunity to conclude that *P. fluorescens* has at least three subpopulations, of which two can be easily distinguished from all other *Pseudomonas* spp. being considered here.

Validation of Framework Sequences by *In Silico* RE Activity on *rrs* Sequences of Organisms Identified as *Pseudomonas* spp.

After the initial segregation of *Pseudomonas* spp. (1,367 isolates) (Supplementary Table 4) on the basis of phylogenetic FWS analysis (represented by 31 sequences including 15 type strains), it is important to validate them. Here, unique RE digestion patterns deduced from the *rrs* sequences of five known *Pseudomonas* spp. provided the necessary evidences. The details of the validation process have been presented as Supplementary Material 1 (Supplementary Tables 6–10).

Nucleotide Motif Analysis for the Validation of Framework Sequences

Additional evidences to further validate the segregation of *Pseudomonas* spp. done on the basis of phylogenetic FWS analysis were collected by the presence of nucleotide motifs (30–50 nts) deduced from isolates of the five known *Pseudomonas* spp. The sequences of 89–414 data sets submitted group-wise to MEME (Multiple EM for Motif Elicitation) program (http://meme.nbcr.net/meme3/meme.html) revealed 45 motifs (15 each of 30, 40 and 50 nts) for each species. To validate the categorization and classification of 1,367 *rrs* sequences belonging to five *Pseudomonas* spp. (Table 3), motifs unique to a particular *Pseudomonas* sp. were identified. A 30 nts unique motif (M15) was found to occur with a very high frequency of (355/375) in *P. aeruginosa rrs* sequences. The 50 nts motifs (M9, M12 and M15) were also found to occur with higher frequency among *P. aeruginosa rrs* sequences (341–352/375). These motifs were found to validate the *rrs* sequences identified as *P. aeruginosa* on the basis of FWS analysis. A search for unique motifs in *rrs* of *P. stutzeri* revealed four motifs viz. M12 (30 nts), M11 (40 nts), M12 (40 nts) and M9 (50 nts), which occurred with high frequency and could be validated on *P. stutzeri* identified on the basis of FWS. Only one motifs M15 (40 nts) could be categorized as unique to *P. putida* and appeared with a frequency of 194/414. Similarly, only one—M12 (40 nts) out of 45 motifs was found to be unique among 89 *P. syringae rrs* sequences. None of the 45 motifs among *rrs* sequences of *P. fluoresens* could be identified as unique (Table 3). However, by exclusion principle, we can separate it from other four *Pseudomonas* spp. used in this study.

Based on two criteria, ten motifs were unique to four *Pseudomonas* spp., and could be validated among *rrs* sequences as follows: 121/219 of *P. aeruginosa*, 171/197 of *P. stutzeri*, 182/347 of *P. putida* and 23/141 of *P. syringae*, identified above on the basis of FWS.

Discussion

Economically important *Pseudomonas* spp. have been equated to a "dumping ground" [8]. It has been suggested to redefine true diversity of *Pseudomonas* [16]. The use of *rpoD* as an alternative to *rrs*, gives poor resolution of *P. fluorescens*, *P. syringae*, *P. entomophila* and *P. putida* [17]. Similarly, *oprD* gene revealed extensive genetic mosaicism [18]. Certain strains of *P. fluorescens* and *P. syringae* yielded low DNA–DNA hybridization (DDH) values between 25 and 39 %, demanding their segregation into the different species. Through studies aimed at reclassifying *Pseudomonas* spp., quite a few have been renamed as: (i) *Comamonas*, (ii) *Acidovorax*, (iii) *Burkholderia* and later as *Ralstonia*, (iv) *Brevundimonas*, and (v) *Stenotrophomonas* [7]. The big issue is the eroding confidence in using *rrs*. Efforts have been made to explore and exploit the hidden features of *rrs* of *Bacillus*, *Clostridium*, *Stentrophomonas* and *Streptococcus* spp. [6, 12–14]. In comparison to these genera, *Pseudomonas* posed a much bigger challenge as around 3,000 of *rrs* sequencing entries, were not classified beyond the genus status.

Restriction Enzyme Sites

A survey of different works reveals that certain REs sites are effective in distinguishing even closely related *rrs* sequences. Around seven different REs—*Alu*I, *Bfa*I, *Dpn*II, *Hae*III, *Rsa*I and *Tru*9I, have been quite instrumental in distinguishing around 2,000 strains belonging to ten species of *Bacillus* spp., 750 strains of 15 species of *Clostridium* spp. and a few strains of *Stenotrophomonas* spp. [6, 12, 13]. In the present study, unique digestion patterns were observed with *Hae*III for *P. aeruginosa*, and with *Alu*I and *Bfa*I for *P. fluorescens*. *P. stutzeri* could be distinguished on the basis of unique digestion pattern achievable with *Tru*9I.

The True *Pseudomonas*

It is worth noting that a large proportion of isolates deposited in the RDP database as *P. aeruginosa*, *P. stutzeri* and *P. syringae* have been properly identified (Table 3). Here, 355/375 *rrs* sequences of *P. aeruginosa*, 190/199 *rrs* sequences of *P. stutzeri* and 68/89 of those belonging to *P. syringae* were found to be supported by FWS and motif analyses. The problem seems to lie with *P. putida* and *P. fluorescens*, where only around 47 % (194/414) of the *rrs* sequences of the former could be validated through motif analysis. This prompts one to conclude that bacterial populations may be reclassified as novel species. The demand for more stringent identification of *P. syringae* is

Table 3 Phylogenetic relationships of *Pseudomonas* sp. with frame work organisms supported by unique motifs

Motif			Frequency of signature									
Nucleotide sequence	Unique no.	Size (nts)	*Pseudomonas* spp.					*Pseudomonas* sp.[a]				
			PAE (375)	PST (199)	PFL (273)	PPT (414)	PSY (89)	PAE (219)	PST (197)	PFL (463)	PPT (347)	PSY (141)
P. aeruginosa												
GCTAATACCGCATACGTCCTGAGGGAGAAA	M15	30	355	0	0	1	0	80	1	0	0	0
ATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACC	M9	50	341	1	0	1	0	119	5	0	0	0
AAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTT	M12	50	347	0	0	1	0	79	1	0	0	0
TGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGA	M15	50	352	1	0	2	0	121	13	0	1	0
P. stutzeri												
TAACGCATTAAGTCGACCGCCTGGGGAGTA	M12	30	3	190	0	6	0	8	171	0	0	0
TTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGT	M11	40	1	134	0	2	0	16	138	0	0	0
TATGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAA	M12	40	0	185	1	6	0	4	165	0	0	0
TGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAG	M9	50	1	133	0	2	0	11	134	0	0	0
P. putida												
AACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACG	M15	40	0	0	2	194	0	0	0	0	182	0
P. syringae												
TAAAGCGCGCGTAGGTGGTTTGTTAAGTTGAATGTGAAAT	M12	40	0	0	3	3	68	0	0	14	0	23
P. fluorescens	No motif could be designated as unique											

[a] Isolates designated as *Pseudomonas* sp. on the basis of FWS (Supplementary Table 3)

PAE: *Pseudomonas aeruginosa*; PFL: *P. fluorescens*; PPT: *P. putida*; PST: *P. stutzeri*; PSY: *P. syringae*

evident as only 23/141 were found to possess unique motifs. Previous studies have shown that *P. fluorescens* has closest affiliation to *P. rhodesiae* and *P. gessardii* [19], such that those *rrs* sequences which fall within the FWS but did not have any motif unique to *P. fluorescens* may actually belong to different species. *P. fluorescens* Pf-5 is so similar to *P. syringae* such that it might be mistaken for the later [17]. *P. syringae* with its high genetic diversity has been proposed to be split into around 17 different species [20]. Significance of our work lies in its potential as a tool for the identification of *Pseudomonas* isolated from diverse geographic locations [21].

The molecular tools developed here can be used to identify *Pseudomonas* spp. and provide multiple makers within a single gene (*rrs*). It may be implied that there is a need to carry out a mega study based on all the genera for which *rrs* sequences have been deposited in the RDP database. This strategy is likely to provide clues and authentic evidences on the location of candidatus phyla whose members are yet to be cultured or identified through metagenomic studies. It will thus fill the gaps in the evolutionary scale.

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