

Multidrug-resistant clinical strains identified as *Pseudomonas putida* group cluster independently of *P. putida* and *Pseudomonas monteilii* type strains in a phylogenetic analysis using eight concatenated hypervariable housekeeping sequences

Kiyoko Tamai^{1, 2)*}, Hiroyuki Hata²⁾, Keiko Tsuchikane³⁾, Shoko Oji³⁾,
Akira Hosoyama³⁾, Koji Sakamoto³⁾, Atsushi Yamazoe³⁾,
Nobuyuki Fujita³⁾ and Takayuki Ezaki²⁾

¹⁾Miroku Medical Laboratory Inc., 659-2 Innai, Saku-shi, Nagano 384-2201, Japan

²⁾Department of Microbiology, Gifu University Graduate School of Medicine, Yanagido 1-1, Gifu 501-1194, Japan

³⁾Biological Resource Center, National Institute of Technology and Evaluation
2-5-8, Katusakamatari, Kisarazu-shi, Chiba 292-0818, Japan

Phylogenetic analysis was performed by using a concatenation of eight full-length polymorphic genes encoding large hypervariable proteins (designated C8HKP) selected from among 121 housekeeping proteins shared and conserved in *Pseudomonas aeruginosa* strain PAO1, *Pseudomonas fluorescens* strain A506, *Pseudomonas putida* NBRC 14164, *Pseudomonas stutzeri* ATCC 17588, and *Pseudomonas syringae* pv. *phaseolicola* strain 1448A, which were selected from phylogenetically different groups. The results were compared with those of a previously reported phylogenetic analysis that used a multilocus sequence analysis (MLSA) incorporating four housekeeping genes (16S rRNA, *gyrB*, *rpoB*, and *rpoD*; designated 4MLSA). Comparison of the substitution rates between C8HKP and 4MLSA revealed that C8HKP was strongly correlated ($R^2=0.897$) with 4MLSA and had twice the resolving power of 4MLSA. Furthermore, the phylogenetic positions of the genus *Pseudomonas* were almost identical between the two methods. These results indicate that C8HKP is suitable for phylogenetic analysis of *Pseudomonas* species. Additionally, C8HKP phylogenetic analysis was performed on draft genome sequences for 33 clinical isolates identified as *P. putida* group by 16S rRNA gene analysis. The results revealed that several strains belonged to three independent clusters in the *P. putida* group. Classification of novel species in the *P. putida* group will therefore be required.

Key words: hypervariable housekeeping proteins, MLSA analysis, multi-drug-resistant *P. putida* group strain, phylogenetic analysis

INTRODUCTION

Members of the genus *Pseudomonas* are known as non-fermenters. This genus includes the largest number of species (more than 200) in the class Gammaproteobacteria, ranging from human pathogens (e.g., *P. aeruginosa* and *P. putida*) and plant pathogens (e.g., *Pseudomonas marginalis* and *P. syringae*) to strains used for bioremediation (e.g., *P. stutzeri*). Several methods, such as lipid composition, SDS-PAGE, and DNA-DNA hybridization, have been used to define the taxonomy of the genus

Pseudomonas. On the basis of these analyses, several new genera, including *Acidovorax* (Willems *et al.*, 1990), *Burkholderia* (Yabuuchi *et al.*, 1992), and *Brevundimonas* (Segers *et al.*, 1994), have been created and transferred from the genus *Pseudomonas*. Moreover, 16S rRNA gene phylogenetic analyses have suggested that several species of the genus *Pseudomonas* should be transferred to other genera (Nohynek *et al.*, 1996; Zhao *et al.*, 1995). On the other hand, several housekeeping genes such as *gyrB*, *rpoD*, and *rpoB* (Ait Tayeb *et al.*, 2005; Yamamoto & Harayama, 1998) have been used for phylogenetic analysis of *Pseudomonas* species because of the poor discrimination power of 16S rRNA-based techniques (Anzai *et al.*, 2000; Moore *et al.*, 1996; Yamamoto *et al.*, 2000). In addition, multilocus sequence analysis

*Corresponding author

E-mail: tamai@miroku-lab.co.jp

Accepted: May 26, 2016

(MLSA) using four housekeeping genes (16S rRNA, *gyrB*, *rpoB*, and *rpoD*) has been proposed for *Pseudomonas* taxonomy (Mulet *et al.*, 2010).

Next-generation sequencing (NGS) has permitted the rapid generation of whole bacterial genome sequences. With the spread of NGS, the number of sequenced bacterial genomes has increased 50 fold or more over the past decade (Land *et al.*, 2015). Furthermore, the possibility of accurate and inexpensive genome analysis has increased the availability of protein sequences for large numbers of housekeeping genes. Here, to improve the reliability of phylogenetic analysis of the genus *Pseudomonas*, we selected suitable hypervariable housekeeping protein sequences for MLSA from the genome information in public databases and compared the results with those obtained by using published MLSA-based phylogenetic analyses. We also determined the phylogenetic positions of *P. putida*-group clinical strains identified by 16S rRNA gene phylogenetic analysis to confirm the usefulness of MLSA using selected hypervariable housekeeping proteins.

MATERIALS AND METHODS

Bacterial strains and data collection

The sample comprised 33 clinical isolates maintained as part of the Japan National Bioresource of Bacterial Pathogen (JNBP) (Table 1). Nucleotide and protein sequence data on *Cellvibrio japonicus* strain Ueda 107 and 147 *Pseudomonas* strains were obtained from the National Center for Biotechnology Information (NCBI) genome database. Table 2 lists the strains and information used in this study.

Draft genome sequences

Draft genomes of the bacterial strains listed in Table 1 were determined by using HiSeq 1000, 2500, or Miseq (Illumina, San Diego, CA, USA) and 454 GS-FLX Titanium (Roche Diagnostics, Basel, Switzerland) sequencing systems. Raw sequence data were assembled by using Newbler version 2.6 (Roche Diagnostics) software. After assembly, more than 90% of the sequences were auto-annotated via the MiGAP (Microbial Genome Annotation Pipeline) program suite version 1.0.50 (Sugawara *et al.*, 2009) (<http://www.migap.org/>). The DDBJ accession numbers for the gene sequences determined in this study are LC151022 to LC151054 (*cca*), LC151055 to LC151087 (*dnaG*), LC151088 to LC151120 (*dnaQ*), LC151121 to LC151153 (*grpE*), LC151154 to LC151186

(*holB*), LC151187 to LC151219 (*holC*), LC151220 to LC151252 (*murB*), and LC151253 to LC151285 (*murF*).

Selection of housekeeping proteins

On the basis of the annotation data for *C. japonicus* strain Ueda 107 and five strains of the genus *Pseudomonas* selected from phylogenetically different groups (*P. aeruginosa* strain PAO1, *Pseudomonas fluorescens* strain A506, *P. putida* NBRC 14164, *P. stutzeri* ATCC 17588, and *P. syringae* pv. *phaseolicola* strain 1448A), we chose 121 housekeeping proteins that were present in all six strains; all of the selected genes were categorized as members of the following classes based on the functional categories of the COG database (<http://www.ncbi.nlm.nih.gov/COG/>): Cell wall/membrane/envelope biogenesis, Cell-cycle control/cell division/chromosome partitioning, Replication/recombination and repair, tRNA modification/translation initiation and elongation, tRNA-synthesis, Coenzyme transport and metabolism, Posttranslational modification/protein turnover/chaperones, Ribosomal proteins, Transcription, or Nucleotide transport and metabolism. The amino acid substitution rates among the homologs of a given protein sequence in the six strains were calculated by using MEGA6 software (Tamura *et al.*, 2013).

Phylogenetic analysis

Selected protein sequences of 148 strains belonging to the genus *Pseudomonas* were obtained from the NCBI genome database and from draft genome information generated in this study. Eight selected protein sequences were concatenated in the following order: Cca-DnaG-DnaQ-GrpE-HolB-HolC-MurB-MurF, yielding a sequence designated C8HKP. Alignment and substitution rates among the strains were calculated by using MEGA6 software. Concatenation of 16S rRNA, *gyrB*, *rpoB*, and *rpoD* genes (4MLSA) was performed as previously described (Mulet *et al.*, 2010). After concatenation, nucleotide sequence alignment was performed and substitution rates determined among the strains by using MEGA6 software in accordance with the procedure for C8HKP. Pearson's correlation coefficient for the substitution rates between C8HKP and 4MLSA was obtained with Microsoft Excel 2007 software. Phylogenetic trees for C8HKP and 4MLSA were prepared by using the neighbor-join-

Table 1 List of the sample comprised 33 clinical isolates

No.	Abbreviation	Strain name	Source	Closest species based on 16S rRNA gene sequence similarity rates (%)	Specimen	Isolation year	Note
1	PPU03	<i>P. putida</i> JNBP 04382	GTC 00055=NBRC 111117	<i>P. japonica</i>	unknown (Human specimen)	1973	
2	PP1	<i>P. putida</i> JNBP 04385	GTC 14033=NBRC 111118	<i>P. plecoglossicida</i>	unknown (Human specimen)	1970	
3	PP2 MDR ^{†*}	<i>P. putida</i> JNBP 04436	GTC 14666=NBRC 111119	<i>P. cremoricolorata</i>	Human urine	2009	MDR strain
4	PP5 MDR	<i>P. putida</i> JNBP 04406	GTC 16335=NBRC 111120	<i>P. taiwanensis</i>	Human urine	2011	MDR strain
5	PP6 MDR	<i>P. putida</i> JNBP 04405	GTC 16336=NBRC 111121	<i>P. montelii</i>	Human sputum	2011	MDR strain
6	PPU04	<i>P. putida</i> JNBP 07332	GTC 10890=NBRC 111122	<i>P. montelii</i>	unknown (Human specimen)	1986	
7	PPU05	<i>P. putida</i> JNBP 07341	GTC 10893=NBRC 111123	<i>P. montelii</i>	unknown (Human specimen)	1986	
8	PPU07	<i>P. putida</i> JNBP 07343	GTC 10897	<i>P. taiwanensis</i>	unknown (Human specimen)	1986	
9	PPU10	<i>P. putida</i> JNBP 04384	GTC 14024=NBRC 111124	<i>P. montelii</i>	unknown (Human specimen)	2008	
10	PPU38 MDR	<i>P. putida</i> JNBP 04435	GTC 14663=NBRC 111125	<i>P. plecoglossicida</i>	Human urine	2009	MDR strain
11	PPU11 MDR	<i>P. putida</i> JNBP 04437	GTC 14667=NBRC 111126	<i>P. taiwanensis</i>	Human urine	2009	MDR strain
12	PPU12 MDR	<i>P. putida</i> JNBP 04438	GTC 14668=NBRC 111127	<i>P. plecoglossicida</i>	Human urine	2009	MDR strain
13	PPU39 MDR	<i>P. putida</i> JNBP 04439	GTC 14669=NBRC 111128	<i>P. taiwanensis</i>	Human pharynx swab	2009	MDR strain
14	PPU13 MDR	<i>P. putida</i> JNBP 04440	GTC 14670=NBRC 111129	<i>P. taiwanensis</i>	Human urine	2009	MDR strain
15	PPU15	<i>P. putida</i> JNBP 04449	GTC 16452=NBRC 111130	<i>P. plecoglossicida</i>	Human sputum	2010	
16	PPU17	<i>P. putida</i> JNBP 04451	GTC 16454=NBRC 111131	<i>P. plecoglossicida</i>	Human bronchial lavage fluid	2010	
17	PPU18	<i>P. putida</i> JNBP 04452	GTC 16455=NBRC 111132	<i>P. plecoglossicida</i>	Human pharynx swab	2010	
18	PPU20	<i>P. putida</i> JNBP 04454	GTC 16457=NBRC 111133	<i>P. montelii</i>	Human urine	2010	
19	PPU21	<i>P. putida</i> JNBP 04455	GTC 16458=NBRC 111134	<i>P. putida</i>	Human pharynx swab	2010	
20	PPU23	<i>P. putida</i> JNBP 04457	GTC 16460=NBRC 111136	<i>P. plecoglossicida</i>	Human urine	2010	
21	PPU27	<i>P. putida</i> JNBP 04461	GTC 16464=NBRC 111139	<i>P. montelii</i>	Human eye discharge	2010	
22	PPU29	<i>P. putida</i> JNBP 04463	GTC 16466=NBRC 111140	<i>P. plecoglossicida</i>	Human sputum	2010	
23	PPU30	<i>P. putida</i> JNBP 04464	GTC 16467=NBRC 111141	<i>P. putida</i>	Human venous blood	2010	
24	PPU32	<i>P. putida</i> JNBP 04466	GTC 16469=NBRC 111142	<i>P. entomophila</i>	Human catheter urine	2010	
25	PPU34	<i>P. putida</i> JNBP 04468	GTC 16471=NBRC 111143	<i>P. taiwanensis</i>	Human urine	2010	
26	PPU36 MDR	<i>P. putida</i> JNBP 04470	GTC 16473	<i>P. montelii</i>	Human clinical	2012	MDR strain
27	PMO05 MDR	<i>P. montelii</i> JNBP 04446	GTC 16480=NBRC 111144	<i>P. taiwanensis</i>	Human sputum	2007	MDR strain
28	PMO06 MDR	<i>P. montelii</i> JNBP 04447	GTC 16481	<i>P. taiwanensis</i>	Human urine	2008	MDR strain
29	PPU37 MDR	<i>P. putida</i> JNBP 04472	GTC 16482	<i>P. plecoglossicida</i>	Human clinical	2012	MDR strain
30	PMO01 MDR	<i>P. montelii</i> JNBP 04441	GTC 16474	<i>P. putida</i>	Human urine	2006	MDR strain
31	PMO02 MDR	<i>P. montelii</i> JNBP 04443	GTC 16476	<i>P. montelii</i>	Human urine	2006	MDR strain
32	PMO03 MDR	<i>P. montelii</i> JNBP 04444	GTC 16477	<i>P. montelii</i>	Human urine	2006	MDR strain
33	PMO04 MDR	<i>P. montelii</i> JNBP 04445	GTC 16479	<i>P. montelii</i>	Human urine	2007	MDR strain

[†]MDR: Multi-Drug Resistant, ^{*} Definition of MDR is as follows: Imipenem $\geq 16 \mu\text{g/ml}$, Amikacin $\geq 32 \mu\text{g/ml}$, Ciprofloxacin $\geq 4 \mu\text{g/ml}$

Table 2 Bacterial strains using genome information

Species	Strain	Accession no. (Assembly)	Type strain or not	Group [†]
<i>Cellvibrio japonicus</i>	Ueda 107	GCA_000019225.1	Type	Outgroup
<i>Pseudomonas alkylphenolia</i>	KL28	GCA_000746525.1	Type	<i>P. putida</i> group
<i>Pseudomonas abietaniphila</i>	NBRC 110664	GCA_000974525.1	Type	<i>P. lutea</i> group
<i>Pseudomonas aeruginosa</i>	MRW44.1	GCA_000282915.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	MPAO1/P1	GCA_000247435.2	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	MPAO1/P2	GCA_000247455.2	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	PAO579	GCA_000296325.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	9BR	GCA_000223925.2	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	213BR	GCA_000223965.2	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	ATCC 14886	GCA_000297275.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	CIG1	GCA_000295475.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	ATCC 25324	GCA_000297295.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	138244	GCA_000215775.4	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	M18	GCA_000226155.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	LESB58	GCA_000026645.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	PACS2	GCA_000168335.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	PABL056	GCA_000290555.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	DQ8	GCA_000283055.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	NCGM1179	GCA_000291745.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	UCBPP-PA14	GCA_000014625.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	CI27	GCA_000297335.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	39016	GCA_000148745.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	NCGM2.S1	GCA_000284555.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	E2	GCA_000482005.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	PA7	GCA_000017205.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas aeruginosa</i>	PAO1	GCA_000006765.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas agarici</i>	NCPPB 2289	GCA_000280785.1	Type	No group assigned
<i>Pseudomonas alcaligenes</i>	NBRC 14159	GCA_000467105.1	Type	<i>P. aeruginosa</i> group
<i>Pseudomonas amygdali</i>	CFBP 3205	GCA_000935645.1	Type	<i>P. syringae</i> group
<i>Pseudomonas avellanae</i>	BPIC 631	GCA_000302915.1	Not Type	<i>P. syringae</i> group
<i>Pseudomonas azotifigens</i>	DSM 17556	GCA_000425625.1	Type	<i>P. stutzeri</i> group
<i>Pseudomonas bauzanensis</i>	W13Z2	GCA_000632535.1	Not Type	<i>P. pertucinogena</i> group
<i>Pseudomonas brassicacearum</i> subsp. <i>brassicacearum</i>	NFM421	GCA_000194805.1	Not Type	<i>P. fluorescens</i> group (<i>P. corrugata</i> subgroup)
<i>Pseudomonas caeni</i>	DSM 24390	GCA_000421765.1	Type	No group assigned
<i>Pseudomonas chlororaphis</i>	O6	GCA_000264555.1	Not Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas chlororaphis</i> subsp. <i>aureofaciens</i>	NBRC 3521	GCA_000813225.1	Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas chlororaphis</i> subsp. <i>aureofaciens</i>	30-84	GCA_000281915.1	Not Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas chlororaphis</i> subsp. <i>chlororaphis</i>	GP72	GCA_000237045.2	Not Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas cichorii</i>	JBC1	GCA_000517305.1	Not Type	<i>P. syringae</i> group
<i>Pseudomonas corrugata</i>	CFBP 5454	GCA_000522485.1	Not Type	<i>P. fluorescens</i> group (<i>P. corrugata</i> subgroup)
<i>Pseudomonas cremoricolorata</i>	NBRC 16634	GCA_000425745.1	Type	<i>P. putida</i> group
<i>Pseudomonas entomophila</i>	L48	GCA_000026105.1	Type	<i>P. putida</i> group
<i>Pseudomonas extremaustralis</i>	14-3 substr. 14-3b	GCA_000242115.2	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas fluorescens</i>	SBW25	GCA_000009225.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)

Table 2 Continued

Species	Strain	Accession no. (Assembly)	Type strain or not	Group †
<i>Pseudomonas fluorescens</i>	NZ007	GCA_000280805.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas fluorescens</i>	BRIP 34879	GCA_000334015.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas fluorescens</i>	A506	GCA_000262325.2	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas fluorescens</i>	SS101	GCA_000263675.2	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas fluorescens</i>	BBc6R8	GCA_000297195.2	Not Type	<i>P. fluorescens</i> group (<i>P. gessardii</i> subgroup)
<i>Pseudomonas fluorescens</i>	R124	GCA_000292795.1	Not Type	<i>P. fluorescens</i> group (<i>P. koreensis</i> subgroup)
<i>Pseudomonas fluorescens</i>	NZ011	GCA_000276585.1	Not Type	<i>P. fluorescens</i> group (<i>P. koreensis</i> subgroup)
<i>Pseudomonas fluorescens</i>	Pf0-1	GCA_000012445.1	Not Type	<i>P. fluorescens</i> group (<i>P. koreensis</i> subgroup)
<i>Pseudomonas fluorescens</i>	NCIMB 11764	GCA_000293885.3	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas fluorescens</i>	Q8r1-96	GCA_000263695.2	Not Type	<i>P. fluorescens</i> group (<i>P. corrugata</i> subgroup)
<i>Pseudomonas fluorescens</i>	Q2-87	GCA_000281895.1	Not Type	<i>P. fluorescens</i> group (<i>P. corrugata</i> subgroup)
<i>Pseudomonas frederiksbergensis</i>	SI8	GCA_000802155.2	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas fulva</i>	NBRC 16637	GCA_000730565.1	Type	<i>P. putida</i> group
<i>Pseudomonas fulva</i>	12-X	GCA_000213805.1	Not Type	<i>P. straminea</i> group
<i>Pseudomonas fuscovaginae</i>	UPB0736	GCA_000251185.1	Not Type	<i>P. fluorescens</i> group (<i>P. asplenii</i> subgroup)
<i>Pseudomonas japonica</i>	NBRC 103040	GCA_000730585.1	Type	<i>P. putida</i> group
<i>Pseudomonas kilonensis</i>	1855-344	GCA_000968575.1	Not Type	<i>P. fluorescens</i> group (<i>P. corrugata</i> subgroup)
<i>Pseudomonas knackmussii</i>	B13	GCA_000689415.1	Type	<i>P. aeruginosa</i> group
<i>Pseudomonas lutea</i>	DSM 17257	GCA_000759445.1	Type	<i>P. lutea</i> group
<i>Pseudomonas mandelii</i>	JR-1	GCA_000257545.3	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas mediterranea</i>	CFBP 5447	GCA_000774145.1	Type	<i>P. fluorescens</i> group (<i>P. corrugata</i> subgroup)
<i>Pseudomonas meliae</i>	CFBP 3225	GCA_000935675.1	Type	<i>P. syringae</i> group
<i>Pseudomonas mendocina</i>	ymp	GCA_000016565.1	Not Type	<i>P. oleovorans</i> group
<i>Pseudomonas mendocina</i>	DLHK	GCA_000287395.1	Not Type	<i>P. oleovorans</i> group
<i>Pseudomonas mendocina</i>	NK-01	GCA_000204295.1	Not Type	<i>P. oleovorans</i> group
<i>Pseudomonas mendocina</i>	NBRC 14162	GCA_000813265.1	Type	<i>P. oleovorans</i> group
<i>Pseudomonas monteilii</i>	NBRC 103158	GCA_000730605.1	Type	<i>P. putida</i> group
<i>Pseudomonas moraviensis</i>	R28-S	GCA_000512275.1	Not Type	<i>P. fluorescens</i> group (<i>P. koreensis</i> subgroup)
<i>Pseudomonas mosselii</i>	DSM 17497	GCA_000621225.1	Type	<i>P. putida</i> group
<i>Pseudomonas oleovorans</i>	MOIL14HWK12	GCA_000510765.1	Not Type	<i>P. oryzihabitans</i> group
<i>Pseudomonas oryzihabitans</i>	NBRC 102199	GCA_000730625.1	Type	<i>P. oryzihabitans</i> group
<i>Pseudomonas otitidis</i>	LNU-E-001	GCA_000632755.1	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas parafulva</i>	NBRC 16636	GCA_000730645.1	Type	<i>P. putida</i> group
<i>Pseudomonas pelagia</i>	CL-AP6	GCA_000410875.1	Type	<i>P. pertucinogena</i> group
<i>Pseudomonas plecoglossicida</i>	NBRC 103162	GCA_000730665.1	Type	<i>P. putida</i> group

Table 2 Continued

Species	Strain	Accession no. (Assembly)	Type strain or not	Group [†]
<i>Pseudomonas poae</i>	RE*1-1-14	GCA_000336465.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas protegens</i>	Pf-5	GCA_000012265.1	Not Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas protegens</i>	CHA0	GCA_000397205.1	Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas</i> "pseudoalcaligenes"	KF707	GCA_000262065.3	Not Type	<i>P. aeruginosa</i> group
<i>Pseudomonas</i> "pseudoalcaligenes"	CECT 5344	GCA_000297075.1	Not Type	<i>P. oleovorans</i> group
<i>Pseudomonas psychrophila</i>	HA-4	GCA_000282975.1	Not Type	<i>P. fluorescens</i> group (<i>P. fragi</i> subgroup)
<i>Pseudomonas psychrotolerans</i>	L19	GCA_000236825.2	Not Type	<i>P. oryzihabitans</i> group
<i>Pseudomonas putida</i>	ND6	GCA_000264665.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	F1	GCA_000016865.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	LS46	GCA_000294445.2	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	B6-2	GCA_000226035.2	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	KT2440	GCA_000007565.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	GB-1	GCA_000019125.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	S16	GCA_000219705.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	HB3267	GCA_000325725.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	NBRC 14164	GCA_000412675.1	Type	<i>P. putida</i> group
<i>Pseudomonas putida</i>	W619	GCA_000019445.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas resinovorans</i>	NBRC 106553	GCA_000412695.1	Type	<i>P. aeruginosa</i> group
<i>Pseudomonas rhizosphaerae</i>	DSM 16299	GCA_000761155.1	Type	No group assigned
<i>Pseudomonas rhodesiae</i>	FF9	GCA_000821225.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas simiae</i>	WCS417	GCA_000698265.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas</i> sp.	Ag1	GCA_000278565.1	Not Type	<i>P. fluorescens</i> group (<i>P. gessardii</i> subgroup)
<i>Pseudomonas</i> sp.	PAMC 25886	GCA_000242655.2	Not Type	<i>P. fluorescens</i> group (<i>P. gessardii</i> subgroup)
<i>Pseudomonas</i> sp.	UW4	GCA_000316175.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas</i> sp.	GM33	GCA_000282295.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas</i> sp.	GM55	GCA_000282395.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas</i> sp.	GM48	GCA_000282335.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas</i> sp.	GM74	GCA_000282455.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas</i> sp.	GM78	GCA_000282475.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas</i> sp.	GM30	GCA_000282275.2	Not Type	<i>P. fluorescens</i> group (<i>P. koreensis</i> subgroup)
<i>Pseudomonas</i> sp.	GM80	GCA_000282515.1	Not Type	<i>P. fluorescens</i> group (<i>P. koreensis</i> subgroup)
<i>Pseudomonas</i> sp.	GM67	GCA_000282435.1	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas</i> sp.	GM60	GCA_000282415.1	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)

Table 2 Continued

Species	Strain	Accession no. (Assembly)	Type strain or not	Group [†]
<i>Pseudomonas</i> sp.	GM102	GCA_000282555.1	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas</i> sp.	GM79	GCA_000282495.1	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas</i> sp.	GM50	GCA_000282375.1	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas</i> sp.	GM18	GCA_000282195.1	Not Type	<i>P. fluorescens</i> group (<i>P. mandelii</i> subgroup)
<i>Pseudomonas</i> sp.	GM17	GCA_000282175.1	Not Type	<i>P. fluorescens</i> group (<i>P. chlororaphis</i> subgroup)
<i>Pseudomonas</i> sp.	M47T1	GCA_000263855.1	Not Type	No group assigned
<i>Pseudomonas</i> sp.	TJI-51	GCA_000190455.3	Not Type	<i>P. putida</i> group
<i>Pseudomonas</i> sp.	GM84	GCA_000282535.1	Not Type	<i>P. putida</i> group
<i>Pseudomonas</i> sp.	Chol1	GCA_000306015.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	DSM 4166 (CMT.9.A)	GCA_000195105.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	T13	GCA_000282955.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	A1501	GCA_000013785.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	ATCC 17588	GCA_000219605.1	Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	XLDN-R	GCA_000280555.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	NF13	GCA_000341615.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	CCUG 29243 (AN10)	GCA_000267545.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	ATCC 14405 (ZoBell)	GCA_000237885.2	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	RCH2	GCA_000327065.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	TS44	GCA_000263395.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	SDM-LAC	GCA_000235745.2	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas stutzeri</i>	DSM 10701 (JM300)	GCA_000279165.1	Not Type	<i>P. stutzeri</i> group
<i>Pseudomonas syringae</i>	DSM 10604	GCA_000597765.1	Type	<i>P. syringae</i> group
<i>Pseudomonas syringae</i> pv. <i>phaeolicola</i>	1448A	GCA_000012205.1	Not Type	<i>P. syringae</i> group
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	B728a	GCA_000012245.1	Not Type	<i>P. syringae</i> group
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	DC3000	GCA_000007805.1	Not Type	<i>P. syringae</i> group
<i>Pseudomonas taeanensis</i>	MS-3	GCA_000498575.1	Type	<i>P. anguilliseptica</i> group
<i>Pseudomonas taiwanensis</i>	DSM 21245	GCA_000425785.1	Type	<i>P. putida</i> group
<i>Pseudomonas thermotolerans</i>	DSM 14292	GCA_000364625.1	Type	<i>P. aeruginosa</i> group
<i>Pseudomonas tolaasii</i>	PMS117	GCA_000276565.1	Not Type	<i>P. fluorescens</i> group (<i>P. fluorescens</i> subgroup)
<i>Pseudomonas toyotomiensis</i>	NBRC 110674	GCA_000974625.1	Not Type	<i>P. oleovorans</i> group
<i>Pseudomonas tuomuerensis</i>	JCM 14085	GCA_000806415.1	Type	<i>P. aeruginosa</i> group
<i>Pseudomonas umsongensis</i>	UNC430CL58Col	GCA_000620285.1	Not Type	<i>P. fluorescens</i> group (<i>P. jessenii</i> subgroup)
<i>Pseudomonas viridiflava</i>	UASWS0038	GCA_000307715.1	Not Type	<i>P. syringae</i> group
<i>Pseudomonas vranovensis</i>	DSM 16006	GCA_000425805.1	Type	<i>P. putida</i> group
<i>Pseudomonas xanthomarina</i>	S11	GCA_000825645.1	Not Type	<i>P. stutzeri</i> group

[†]This category are defined in Mulet *et al.*, 2010

ing method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in a bootstrap test (500 replicates) is shown next to each branch (Felsenstein, 1985). Evolutionary distances were computed by using the Tamura-Nei method (Tamura & Nei, 1993) for 4MLSA and the Dayhoff matrix-based method

(Schwarz & Dayhoff, 1978) for C8HKP and were documented in units of number of base substitutions per site.

RESULTS AND DISCUSSION

Selecting the top eight hypervariable proteins from among the housekeeping proteins

We selected 121 housekeeping proteins from the genomes of *C. japonicus* strain Ueda 107 and five *Pseudomonas* species selected from phylogenetically different groups. To select hypervariable housekeeping proteins, we calculated the average amino acid substitution rate among the above six species (Table 3). Whereas the average substitution rates of the 16S rRNA gene and 4MLSA were 5.7% and 16.6% respectively, the average substitution rate based on the housekeeping proteins ranged from 12.1% to a high of 39.0%. This result suggested that many of the housekeeping proteins had the potential for highly discriminative phylogenetic analysis. On the basis of these results, we utilized categories that had more than a 16.6% average substitution rate (i.e. the average substitution rate of 4MLSA). We selected eight hypervariable housekeeping proteins (CCA, DnaG, DnaQ, GrpE, HolB, HolC, MurB, and MurF) that were present in all genomes analyzed and exhibited relatively low deletion and insertion rates (Table 4).

Phylogenetic analysis of C8HKP and 4MLSA

The amino acid substitution rates among *Pseudomonas* species and the substitution rates between these *Pseudomonas* species and *C. japonicus* were analyzed on the basis of a concatenation of eight hypervariable housekeeping proteins (C8HKP) encoded by housekeeping genes selected from the genomic data of 147 strains of 62 *Pseudomonas* spe-

cies and one strain of *C. japonicus* strain Ueda 107. We conducted a correlation analysis of the substitution rates estimated on the basis of C8HKP and those estimated on the basis of 4MLSA. A strong correlation was observed between the two sets of substitution rates ($R^2=0.897$). Furthermore, the resulting slope of the correlation equation (1.86) indicated that C8HKP had approximately twice the resolving power of 4MLSA in terms of phylogenetic analysis (Fig. 1). The phylogenetic tree generated by using the neighbor-joining method also indicated that C8HKP had approximately twice the resolving power of 4MLSA (Fig. 2). The phylogenetic positions of each of the *Pseudomonas* strains were almost identical between 4MLSA and C8HKP. The positions of *Pseudomonas pseudoalcaligenes* KF707, *Pseudomonas resinovorans* NBRC 106553, and *Pseudomonas thermotolerans* DSM 14292 were included in the *P. aeruginosa* group through 4MLSA. However, they formed independent clusters with *Pseudomonas taeanensis* MS-3, *Pseudomonas fulva* 12-X, *Pseudomonas alcaligenes* NBRC 14159, and *Pseudomonas oleovorans* group through C8HKP. *Pseudomonas tuomuensis* JCM 14085 was also unclustered by C8HKP, regardless of its association with the *P. aeruginosa* group by 4MLSA (Fig. 2). Examination of the *P. putida* group revealed that the phylogenetic positions of C8HKP were identical to those of 4MLSA (Fig. 3). These data suggested that phylogenetic analysis of C8HKP provides more reliable information than does analysis of 4MLSA for *Pseudomonas* taxonomy, especially in the *P. putida* group.

Table 3 Amino acid substitution rates of housekeeping proteins

Category	Number of proteins	Average substitution rate among six strains
Cell wall/membrane/envelope biogenesis	3	39.0%
Cell cycle control, cell division, chromosome partitioning	3	34.3%
Replication, recombination and repair	17	24.3%
tRNA modification/translation initiation and elongation	9	22.4%
tRNA-synthesis	20	22.1%
Coenzyme transport and metabolism	2	21.5%
Posttranslational modification, protein turnover, chaperones	5	18.5%
Ribosomal proteins	53	14.0%
Transcription	7	13.2%
Nucleotide transport and metabolism	2	12.1%
16S rRNA gene [†]		5.7%
4MLSA (16S rRNA- <i>gyrB-rpoD-rpoB</i>) [†]		16.6%

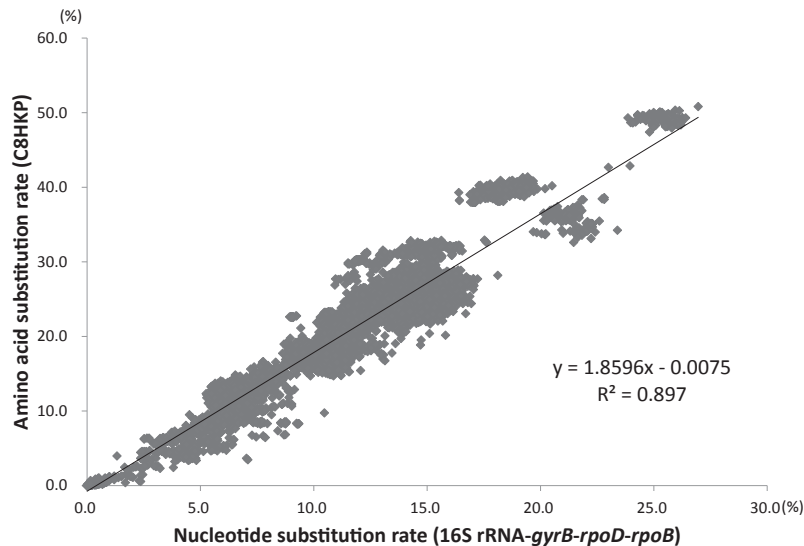
[†] Average substitution rates of 16S rRNA gene and 4MLSA (16S-*gyrB-rpoB-rpoD*) were nucleotide-based.

Table 4 Selected hypervariable housekeeping proteins for MLSA of genus *Pseudomonas*

Gene symbol [†]	Gene product [†]	Category [‡]	Average substitution rate among six strains	Average amino acid length	C.V. (length)
<i>holC</i>	DNA polymerase III, chi subunit	Replication, recombination and repair	47.7%	141	1.1%
<i>murB</i>	UDP-N-acetylenolpyruvoyl-glucosamine reductase	Cell wall/membrane/envelope biogenesis	38.7%	339	0.1%
<i>murF</i>	UDP-N-acetylmuramyl pentapeptide synthase	Cell wall/membrane/envelope biogenesis	36.5%	458	0.6%
<i>holB</i>	DNA polymerase III, delta prime subunit	Replication, recombination and repair	34.8%	330	1.1%
<i>dnaG</i>	DNA primase (bacterial type)	Replication, recombination and repair	32.3%	663	2.5%
<i>cca</i>	tRNA nucleotidyltransferase/poly(A) polymerase	tRNA modification/translation initiation and elongation	32.1%	405	5.8%
<i>grpE</i>	Molecular chaperone GrpE (heat shock protein)	Posttranslational modification, protein turnover, chaperones	32.1%	187	1.2%
<i>dnaQ</i>	DNA polymerase III, epsilon subunit or related 3'-5' exonuclease	Replication, recombination and repair	28.5%	247	3.2%

[†]Gene symbols and gene product used for orthologous sequences are consistently those used in the *P. aeruginosa* PAO1 genome annotation.

[‡]This category is same as Table 3.

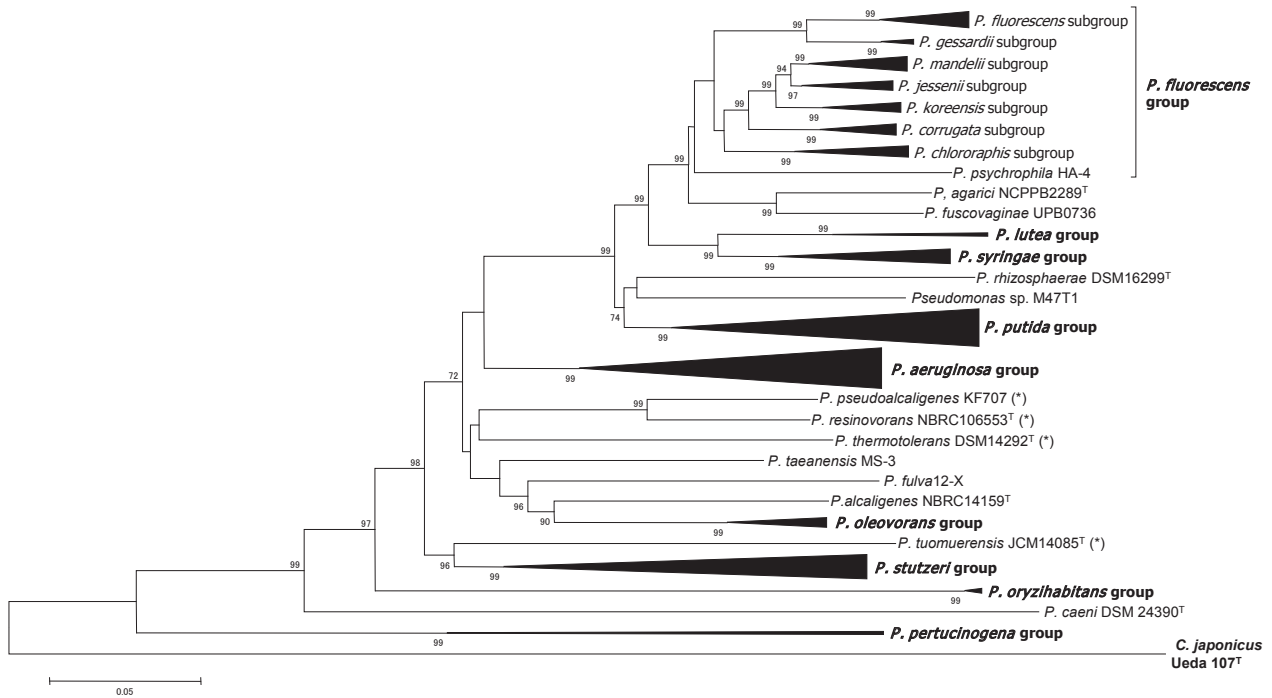
**Fig. 1 Relationship between 4MLSA and C8HKP substitution rates**

Each dot represents a pairwise comparison between 4MLSA substitution rates and C8HKP substitution rates. Both distances were calculated in a round-robin manner by using 147 nucleotides (4MLSA) or 147 proteins (C8HKP).

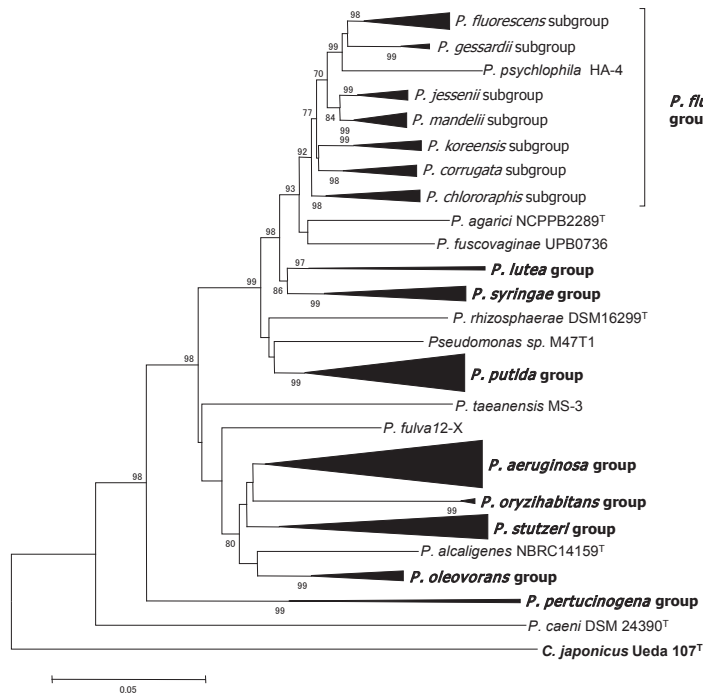
Phylogenetic analysis of clinical *P. putida* isolates by using C8HKP

We performed a C8HKP-based phylogenetic analysis of the draft genome sequences of 33 clinical isolates (Fig. 4). Despite previous identification by 16S

rRNA gene analysis (Fig. 5), several strains from among the *P. putida* group-type species formed three independent clusters, and these clusters clearly differed from *P. putida* group-type species. In these three independent clusters, 11 of the 16 multi-

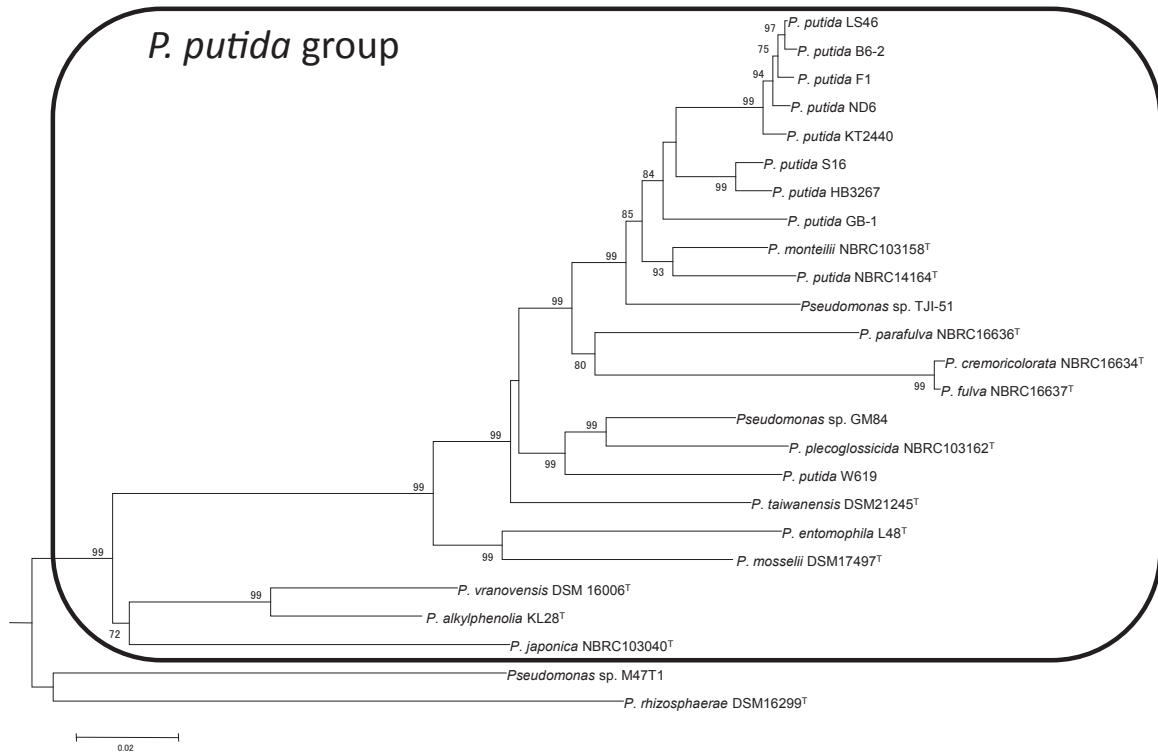


a: C8HKP

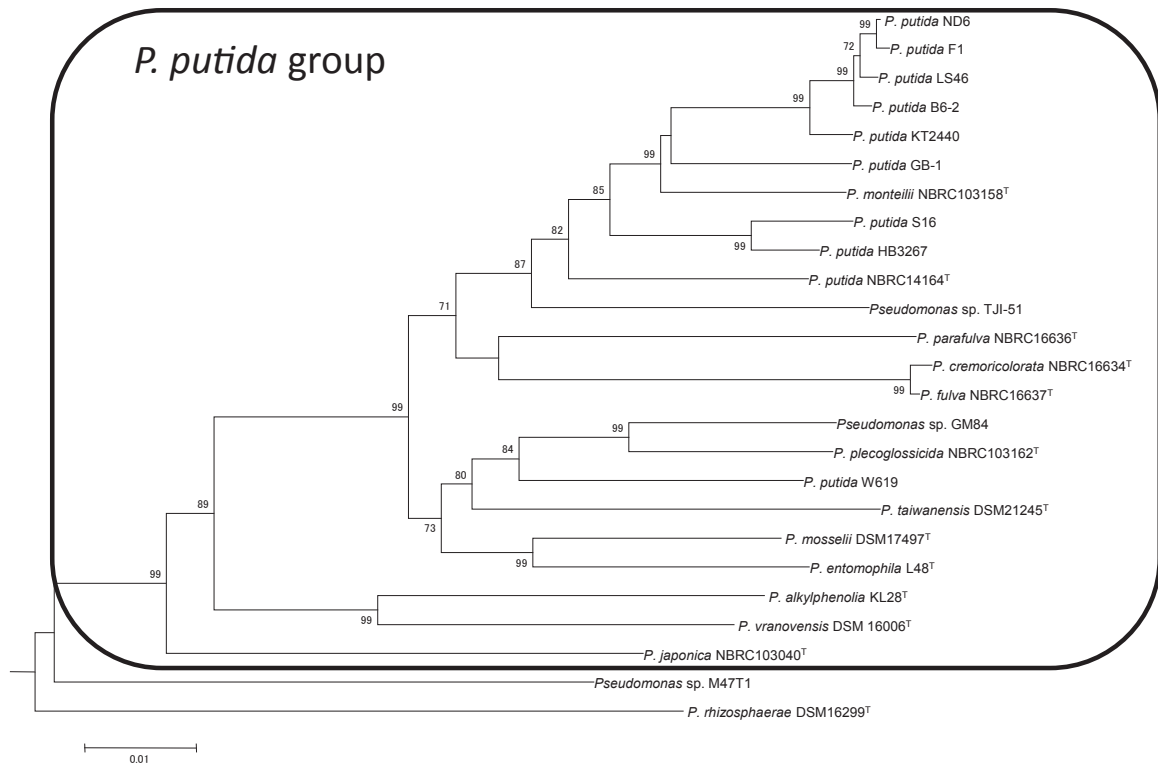


b: 4MLSA

Fig. 2 C8HKP-based (a) and 4MLSA-based (b) phylogenetic trees of members of the genus *Pseudomonas* Phylogenetic trees from C8HKP and 4MLSA were prepared by using the neighbor-joining method (Saitou & Nei, 1987). The analysis involved 147 nucleotide sequences (4MLSA) and 147 proteins (C8HKP). All positions containing gaps and missing data were eliminated. There were a total of 3257 positions for 4MLSA and 2515 positions for C8HKP in the final datasets. Only bootstrap values >70% are shown. *Cellvibrio japonicus* Ueda 107^T was used as an outgroup. Asterisk (*) strains in C8HKP are those found to belong to the *P. aeruginosa* group in 4MLSA.



a: C8HKP



b: 4MLSA

Fig. 3 Expanded subtrees of the *Pseudomonas putida* group in the C8HKP-based (a) and 4MLSA-based (b) phylogenetic trees. These trees were expanded subtrees of the *P. putida* group in the C8HKP-based (a) and 4MLSA-based (b) phylogenetic trees. Analytical conditions were the same as those in Fig. 2.

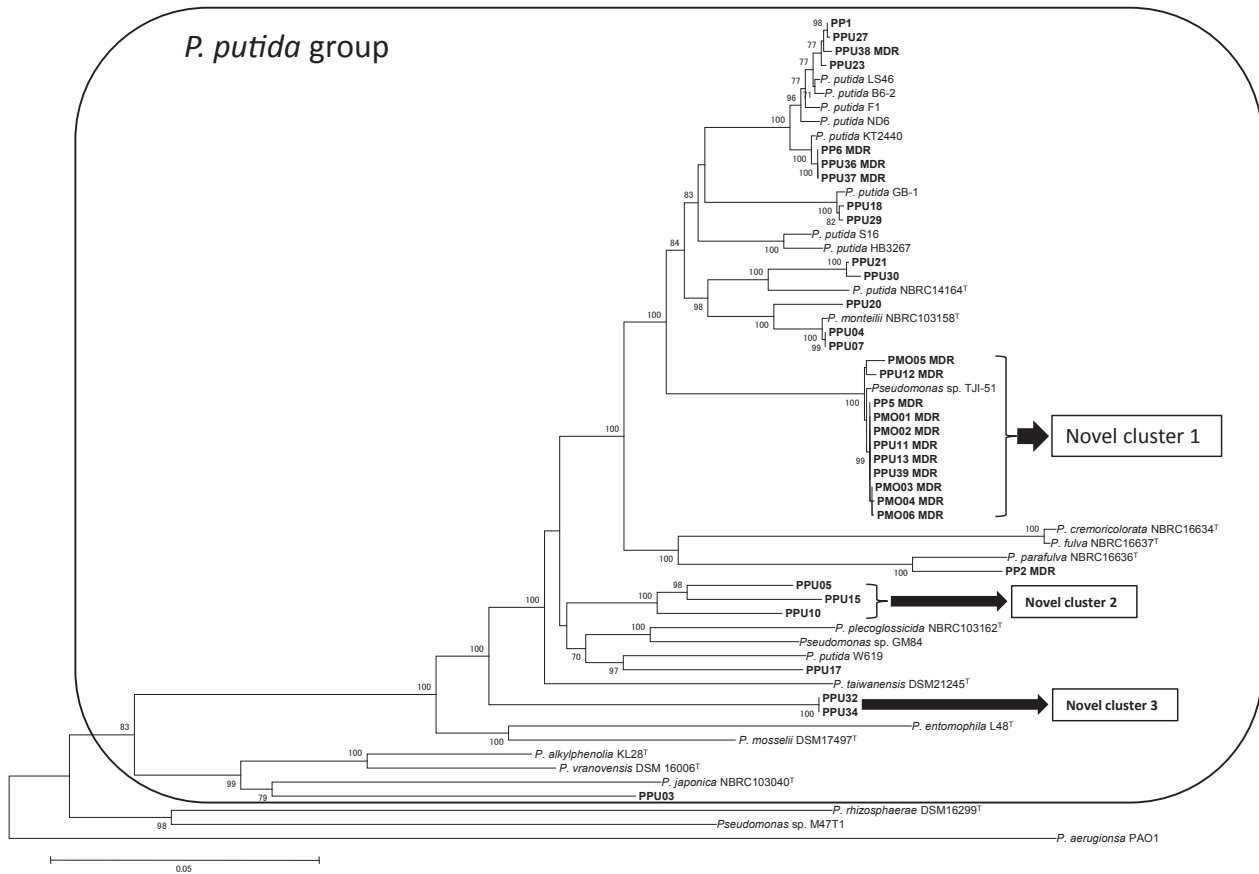


Fig. 4 C8HKP-based phylogenetic tree of *Pseudomonas putida* group, including clinical isolates

The phylogenetic tree of C8HKP was prepared by using the neighbor-joining method (Saitou & Nei, 1987). The analysis involved 59 protein sequences. All positions containing gaps and missing data were eliminated. There were a total of 2645 positions in the final datasets. Only bootstrap values >70% are shown. *Pseudomonas aeruginosa* PAO1 was used as an outgroup.

drug-resistant (MDR) strains with *Pseudomonas* sp. TJI-51 formed a single cluster. This cluster was clearly distant from the type strains of both *P. putida* and *Pseudomonas monteilii*. These results suggest that these 11 strains represent a novel species. Further investigations (i.e. of biochemical characteristics) will be needed before these strains can be proposed as new species.

Some of the strains suspected as being novel species (PMO01, PMO02, PMO03, PMO04, PMO05, and PMO06) were identified as MDR *P. aeruginosa* by using the MicroScan WalkAway Plus and MicroScan autoSCAN-4 (Beckman Coulter, Inc., Brea, CA, USA) phenotype-based identification system. Saiman *et al.* (2003) also reported that some strains of *P. putida*/*P. fluorescens* isolated from patients with cystic fibrosis were misidentified as *P. aeruginosa* by the MicroScan Autoscan system (Beckman Coulter, Inc.).

These findings suggest that, especially in the case of MDR strains, clinically isolated *P. aeruginosa* strains may contain *P. putida*-group strains. Sequence-based identification is still difficult for clinical laboratories, and an easier phenotype-based method for distinguishing *P. putida* group from *P. aeruginosa* is needed.

ACKNOWLEDGMENTS

This work was performed as part of a project supported by the Ministry of Economy, Trade and Industry of Japan.

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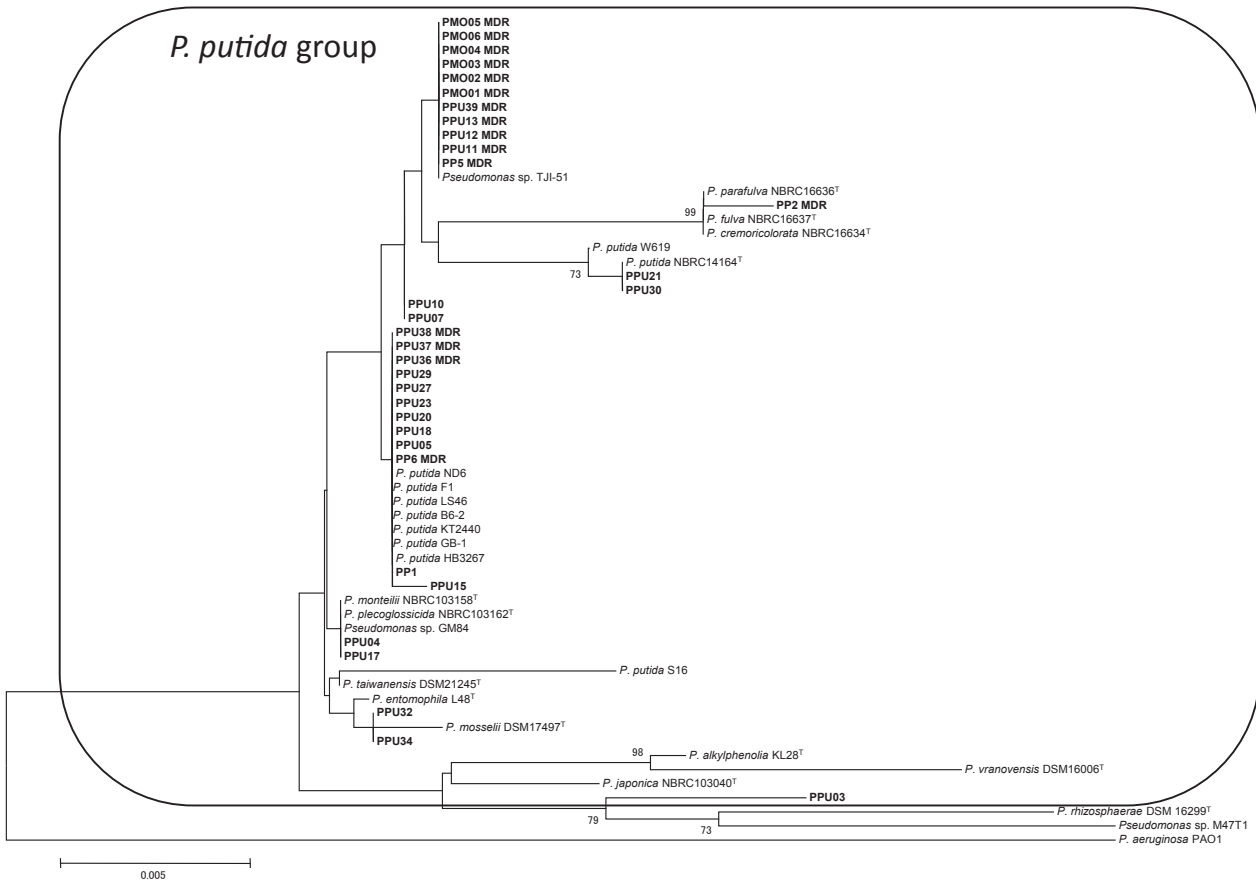


Fig. 5 16S rRNA gene phylogenetic tree of 33 *Pseudomonas putida* group clinical isolates

The phylogenetic tree of the 16S rRNA gene was prepared by using the neighbor-joining method (Saitou & Nei, 1987). The analysis involved 59 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1107 positions in the final datasets. Only bootstrap values >70% are shown. *Pseudomonas aeruginosa* PAO1 was used as an outgroup.

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Pseudomonas putida group の多剤耐性臨床分離株は、高度可変的な 8 個の housekeeping 遺伝子配列の連結を用いた系統解析を行うと、*P. putida* および *Pseudomonas monteilii* の基準株とは独立したクラスターを形成する

玉井清子^{1,2)}, 波多宏幸²⁾, 土金恵子³⁾, 黄地祥子³⁾, 細山 哲³⁾, 阪本康司³⁾, 山副敦司³⁾, 藤田信之³⁾, 江崎孝行²⁾

¹⁾ 株式会社ミロクメディカルラボラトリー, ²⁾ 岐阜大学大学院医学系研究科病原体制御学分野,

³⁾ 製品評価技術基盤機構バイオテクノロジーセンター

Pseudomonas 属の主要 5 菌種に共通に保存されている 121 個の housekeeping proteins より、高い配列多型をもつ 8 種類の蛋白配列を選択した。これらの配列を結合した配列 (C8HKP) を用い、既報の 4MLSA (16S rRNA, *gyrB*, *rpoB*, *rpoD*) を用いた系統解析法と比較した。両者の配列置換率を比較した結果、相関係数は $R^2=0.897$ と高い相関を認め、C8HKP は約 2 倍の解像度を持つことが明らかとなった。加えて、系統樹を比較した結果、各菌種の系統位置は両者で概ね一致していた。このことから、C8HKP による系統解析は *Pseudomonas* 属の系統分類に有用であることが示唆された。さらに、*Pseudomonas putida* group に同定された多剤耐性株を含む臨床分離株 33 株のドラフトゲノム配列を決定し、C8HKP による系統解析を行ったところ、*P. putida* および *Pseudomonas monteilii* の基準株とは独立したクラスターが複数認められ、多剤耐性株はこのクラスターの一つに集中して認められた。