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EXPERIMENTAL ARTICLES

Taxonomic Heterogeneity of the Collection Strains of Fluorescent Pseudomonads

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Abstract—Typing of 13 strains of fluorescent pseudomonads from the Belarusian collection of nonpathogenic microorganisms (BIM) by ERIC-PCR and BOX-PCR revealed high level of genetic heterogeneity in bacteria, most of which have been previously identified as *Pseudomonas fluorescens* according to the classical scheme. Evaluation of the similarities of the 16S rRNA gene sequences and their phylogenetic analysis excluded affiliation of the bacteria under study within the same species and allowed them to be distributed within three relatively distant clusters of the genus *Pseudomonas* phylogenetic tree. While eight strains fell into the phylogenetic group of *P. fluorescens,* only one of them could be identified as *P. fluorescens.* Four strains clustered within the *P. vancouverensis* phylogenetic group, formed by new species, which have been described mainly according to the evaluation of genome relationships. One bacterium was related to a stable branch that did not contain any type strains of the known *Pseudomonas* spp. These results indicate taxonomic heterogeneity of collection strains of the fluorescent pseudomonads and demonstrate the necessity of identification of them considering the requirements of phylogenetic bacterial taxonomy.

Keywords: Pseudomonas, genotyping, phylogeny, 16S rRNA gene, identification.

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Fluorescent pseudomonads are widespread in nature and are easily isolated from various ecological niches. Traditionally, these bacteria have been attributed to saprophytic species of the first rRNA homology group of *Pseudomonas fluorescens* and *P. putida* [1]. Since the first description of these species at the end of the 19th century, many scientifically interesting strains identified as *P. fluorescens* and *P. putida* have been registered in a number of collections. However, prior to the development of the phylogenetic principles of bacterial classification, taxonomists had no unified opinion regarding the taxonomical status of these two species. While some authors questioned the possibility of reliable distinction between these species and considered them as two forms of one species, the heterogeneity of bacteria referred to as *P. fluorescens* and *P. putida* was obvious [2]. This heterogeneity resulted in subdivision of these species into biovars, actually corresponding to the status of subspecies [3]. Nevertheless, the classical scheme did not solve all the problems of identification of the fluorescent pseudomonads. In many cases, scrupulous analysis of the results obtained using this scheme induced researchers to conclude that the bacteria in question belonged not to the *P. fluorescens* and *P. putida* species proper, but rather to the corresponding phenotypic groups [4].

Phylogenetic revision based on comparative analysis of nucleotide sequences of 16S rRNA genes [5–7] excluded a number of previously attributed species from the genus *Pseudomonas*. As a consequence, many novel taxonomical groups of various ranks were established in different classes of *Proteobacteria*. However, due to the description of new species and, to a less extent, to the revision of other taxa, the number of species belonging to *Pseudomonas* increased continually. At present, the list of prokaryotic names at www.bacterio.cict.fr contains over 110 valid *Pseudomonas* genomospecies (not including subspecies). According to Anzai et al. [7], the genus *Pseudomonas* is subdivided into seven phylogenetic groups, including the *P. fluorescens* group that comprises 15 species and the *P. putida* group that comprises 5 species. In the phylogenetic tree, the *P. fluorescens* and *P. putida* groups are not most closely related, but are rather separated by several branches constituted by the species not belonging to these groups. Comparative phylogenetic analysis of the *gyrB* and *rpoD* sequences also demonstrated that *P. fluorescens* and *P. putida* groups were rather distant, although some strains of the *P. putida* phenotypic group clustered with the species of the *P. fluorescens* phylogenetic group [8].

Thus, it could be suggested that many bacteria previously deposited in collections as *P. fluorescens* and *P. putida* actually are genetically heterogeneous and, in accordance with the rules of modern phylogenetic

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Table 1. Collection strains of fluorescent pseudomonads used in this study

Taxonomic position according to the BIM catalogue [9]	Strain designation and number	References
Pseudomonas fluorescens biovar I	BIM B-98 (=VKM B-1476 = No. 11)	[10]
	BIM B-100 (=VKM B-1459 = C-5-5)	[4, 10]
	BIM B-143 (=R-3)	[4]
	BIM B-158 (=VKM B-1461 = 5-2)	[4, 10]
P. fluorescens biovar IV	BIM B-187 (=R-30)	[9]
P. fluorescens biovar V	BIM B-86 (=*P. syringae* pv. *syringae* 345)	[4, 12]
	BIM B-99 (=VKM B-1471 = No. 1)	[10]
	BIM B-147 (=R-14)	[4]
	BIM B-149 (=R-18)	[9]
	BIM B-156 (=VKM B-1472 = 2)	[10]
	BIM B-222 (=R-80)	[4]
P. putida	BIM B-228 (=M-3)	[4]
Pseudomonas sp.	BIM B-137 (=*P. fluorescens* B No. 19)	[4, 11]

taxonomy, do not belong to these species. The necessity for verification of the taxonomic position of the strains of fluorescent pseudomonads by molecular techniques is obvious. The goal of this work was to evaluate the heterogeneity of a number of collection strains from the *P. fluorescens–P. putida* group by genotyping and to determine their phylogenetic position by comparative analysis of 16S rRNA gene sequences.

MATERIALS AND METHODS

Thirteen strains of fluorescent pseudomonads from the Belarusian collection of nonpathogenic microorganisms (BIM), some of which have been also deposited in the All-Russian Collection of Microorganisms (VKM), were used throughout this study (Table 1). The strains were cultivated in TY medium [13].

Genotyping of the bacteria was performed using ERIC-PCR and BOX-PCR as described earlier [13].

Sequencing of 16S rRNA genes and analysis of the sequences were carried out as described in [13].

RESULTS AND DISCUSSION

Rep-PCR genotyping. To determine the genetic heterogeneity and to reveal the related groups of the studied strains, Rep-PCR genotyping was carried out using ERIC1R, ERIC2, and BOXA1R primers. Fingerprints of BIM B-98–BIM B-158 and BIM B-99–BIM B-156 pairs showed high similarity or even identity, irrespective of the primer used (Fig. 1). Significant similarities were found between the fingerprints of strains BIM B-143 and BIM B-147. In all other cases, it was difficult or impossible to determine the similarity between the fingerprints even in the individual bands of the amplified fragment patterns. Thus, genotyping revealed three groups (pairs) of related bacteria; however, no relationships were found between these groups, between any of the groups and bacteria not included into this group, or between any individual bacteria outside the pairs. Taking into account the resolution of Rep-PCR [14], one could affirm that bacteria within each of the revealed pairs belong to the same species. In general, the results of genotyping showed the studied bacteria to be genetically heterogeneous, indicating their taxonomical diversity.

Sequencing of 16S rRNA genes and sequence analysis. Nucleotide sequences (over 1400 bp) of 16S rRNA genes from all the strains were determined and submitted to the GenBank database (accession nos. GU784930–GU784942). A BLAST search in the GenBank database revealed that the 16S rRNA genes from all the analyzed bacteria are most similar to the corresponding genes from members of the genus *Pseudomonas*. Sequences of 16S rRNA genes from the type strains of the supposedly most closely related species (according to BLAST search) and of several intensely studied rhizosphere strains of *P. fluorescens* were selected for pairwise comparison, the results of which are shown in Table 2.

Comparative pairwise analysis indicated maximal similarity of 16S rRNA sequences between the strains BIM B-143, BIM B-147, and BIM B-187 and the type strain *P. fluorescens* ATCC 13525^T (accession no. AF094725). The GenBank database contains three significantly different 16S rRNA gene sequences of the *P. fluorescens* type strain. Only two of them are supported by references to publications on taxonomy of pseudomonads; the accession numbers are D84013 [5] and Z76662 [6]. The 16S rRNA sequences from BIM B-143, BIM B-147, and BIM B-187 share much less similarity with these sequences than with the sequence AF094725 and with the corresponding genes of the type strains of *P. salomonii* and *P. trivialis*. The differences between the versions of 16S rRNA gene sequences of *P. fluorescens* type strain are most probably due to sequencing errors (for example, the D84013 sequence contains two ambiguously determined and

six undetermined nucleotides). Differences between copies of 16S rRNA genes in the genome of *P. fluorescens* type strain also could not be excluded as a possible reason.

Strain BIM B-100 was close to strains BIM B-143, BIM B-147, and BIM B-187 in the similarity values of 16S rRNA gene sequences in pairwise comparisons; however, it exhibited the greatest similarity to the type strain of *P. orientalis*.

The determined nucleotide sequence of the 16S rRNA gene from BIM B-158 (1429 bp) was identical to that of BIM B-98 (1440 bp). Maximal similarity was found between these sequences and 16S rRNA genes of the type strains of *P. cedrina* subsp. *cedrina* and *P. cedrina* subsp. *fulgida*.

The 16S rRNA gene sequences of the strain BIM B-137 shared more than 99% of identity with those of the type strains of *P. veronii, P. trivialis*, and *P. antarctica*.

The similarity of 16S rRNA gene sequences of BIM B-149 and the *P. grimontii* type strain was 99.9%; somewhat smaller values were obtained in comparisons with type strains of *P. rhodesia* and *P. marginalis*.

The sequences of 16S rRNA genes of BIM B-86, BIM B-99, and BIM B-156 were most similar to those of the type strains of *P. moraviensis, P. koreensis, P. jessenii, P. reinekei*, and the intensely studied *P. fluorescens* Pf0-1; however, only BIM B-156 shared 99% or greater similarity.

BIM B-222 demonstrated maximal similarity of 16S rRNA gene sequences with the intensely studied bacteria *P. fluorescens* Pf-5 and *P. fluorescens* CHA0. The level of similarity with the most related type strains of the known *Pseudomonas* spp. was a little above 98%.

Phylogenetic analysis of 16S rRNA genes demonstrated that the studied and reference bacteria constituted three large robust clusters (Fig. 2). Two of them corresponded to the phylogenetic groups of *P. fluorescens* and *P. syringae*, which were previously established by Anzai et al. [7]. The third cluster (the *P. vancouverensis* phylogenetic group) comprised type strains of the species which have not been described at the moment of the last phylogenetic revision of the genus *Pseudomonas*. Importantly, the bacteria which were found to be related according to genotyping results formed common branches in the phylogenetic tree, which were especially stable (bootstrap value 98) for strains with a high level of fingerprint identity (BIM B-98–BIM B-158 and BIM B-99–BIM B-156 pairs).

Most of the strains (eight) belonged to the *P. fluorescens* group. Only one of them, BIM B-187, belonged to the branch of *P. fluorescens*. This species was represented in the analysis by three versions of the type strain 16S rRNA gene sequences obtained from GenBank. As mentioned above, there are essential differences between these versions (Table 2). Nevertheless, the cluster of *P. fluorescens* was highly stable (bootstrap value 71). The branch that included BIM B-143, BIM B-147, and the *P. salomonii* type strain was found to be closest to the *P. fluorescens* cluster.

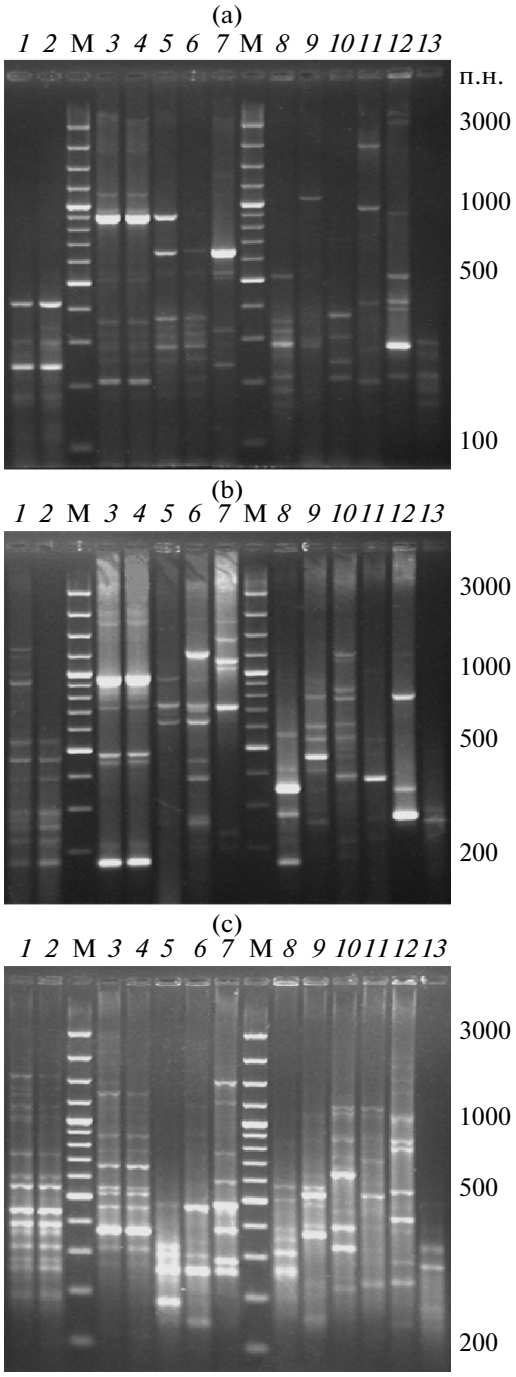

Fig. 1. Genotyping of fluorescent pseudomonads by Rep-PCR using ERIC1R (a), ERIC2 (b) and BOXA1R (c) primers. Lanes: BIM B-158 (*1*), BIM B-98 (*2*), BIM B-156 (*3*), BIM B-99 (*4*), BIM B-143 (*5*), BIM B-147 (*6*), BIM B-187 (*7*), BIM B-86 (*8*), BIM B-100 (*9*), BIM B-137 (*10*), BIM B-149 (*11*), BIM B-228 (*12*), and BIM B-222 (*13*); M, molecular mass marker, Gene Ruler^{TM} 100 bp Plus DNA Ladder (Fermentas, Lithuania).

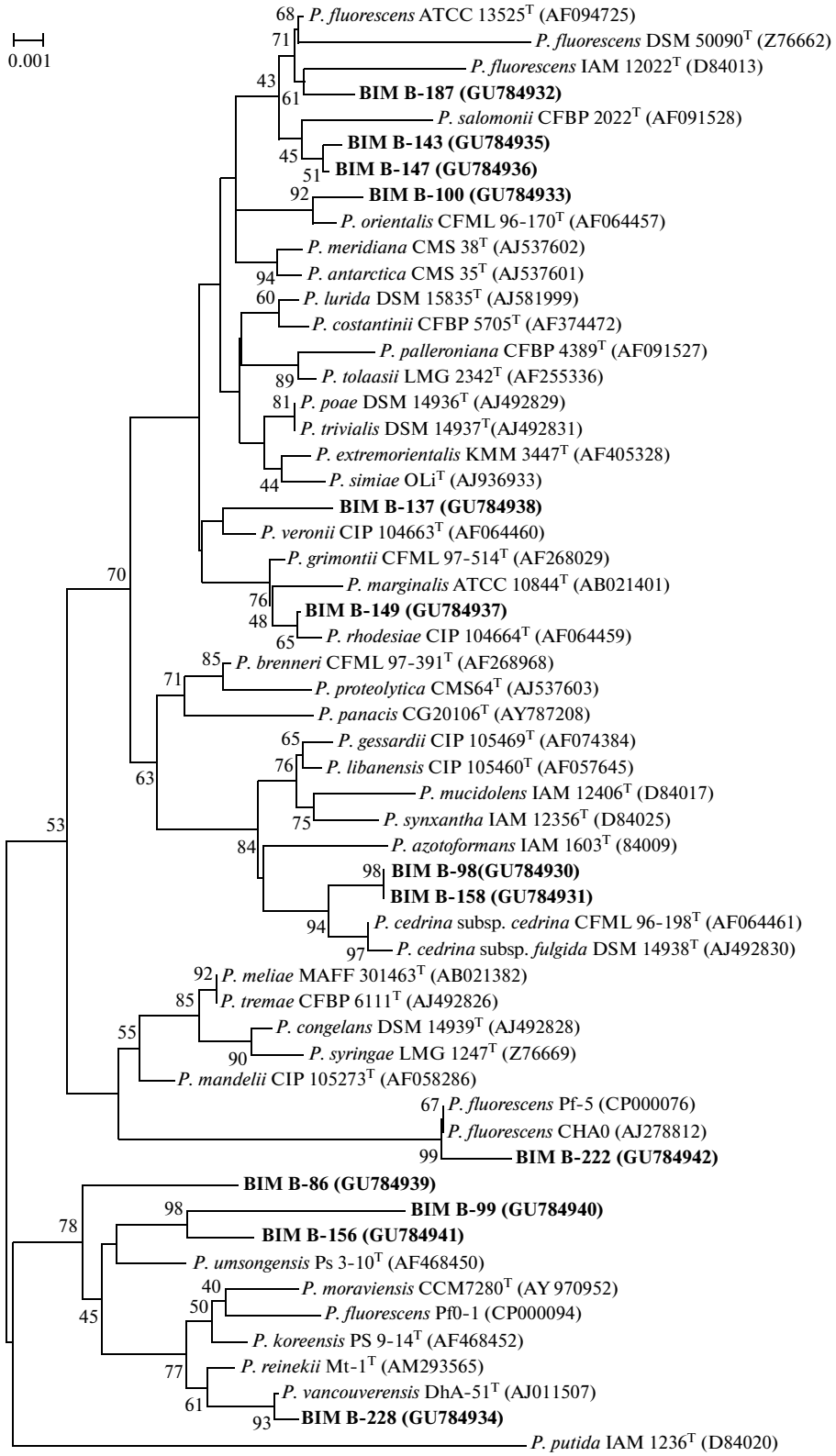

Fig. 2. Phylogenetic tree showing relations between the fluorescent pseudomonads from BIM collection and bacteria of the genus *Pseudomonas*. The GenBank(www.ncbi.nlm.nih.gov/genbank/) accession numbers of the sequences are shown in parentheses. There are 1299 nucleotides in the comparison dataset. Bootstrap values were calculated based on 1000 resamplings. Scale bar, 0.001 substitutions per nucleotide position.

Table 2. Percent similarity of 16S rRNA gene nucleotide sequences of the fluorescent pseudomonads from BIM collection and most closely related type and reference strains from the GenBank database

Bacteria (GenBank accession nos.)	B-143	B-147	B-187	B-100	B-98	B-158	B-137	B-149	B-86	B-99	B-156	B-228	B-222
P. fluorecsens ATCC 13525^T (AF094725)	[1]**99.6**	**99.7**	**99.7**	**99.3**	98.9	98.8	98.8	99.3	97.8	96.7	97.5	97.1	97.9
P. fluorecsens IAM 12022^T (D84013)	99.0	99.1	99.2	98.8	98.3	98.3	98.3	98.8	97.3	96.3	97.0	97.0	96.7
P. fluorecsens DSM 50090^T (Z76662)	98.2	98.4	98.5	98.1	97.6	97.6	97.6	98.1	96.5	95.7	96.3	96.3	96.0
P. salomonii CFBP 2022^T (AY091528)	**99.3**	**99.2**	99.1	98.6	98.2	98.2	98.3	98.7	97.9	96.9	97.6	97.6	97.5
P. trivialis DSM 14937^T (AJ492831)	**99.3**	**99.4**	**99.5**	**99.3**	98.5	98.5	**99.1**	99.5	97.6	96.6	97.3	97.4	97.2
P. orientalis CFML 96-170^T (AF064457)	99.0	99.1	**99.2**	**99.7**	99.1	99.1	98.8	99.2	97.6	96.6	97.3	97.4	97.1
P. antarctica CMS 35^T (AJ537601)	99.1	**99.2**	**99.2**	**99.4**	98.7	98.6	**99.1**	99.5	97.7	96.7	97.3	97.4	97.3
P. cedrina subsp. *cedrina* CFML 96-198^T (AF064461)	98.3	98.4	98.5	98.8	**99.7**	**99.7**	98.0	98.6	97.6	96.8	97.5	98.3	96.3
P. cedrina subsp. *fulgida* DSM 14938^T (AJ492830)	98.2	98.3	98.4	98.7	**99.7**	**99.7**	97.9	98.5	97.5	96.7	97.4	98.3	96.2
P. gessardii CIP 105469^T (AF074384)	98.4	98.4	98.6	98.4	**99.4**	**99.4**	98.3	98.9	97.7	96.7	97.4	98.4	96.6
P. veronii CIP 104663^T (AF064460)	98.8	98.9	99.0	99.1	98.4	98.3	**99.2**	99.4	98.0	96.9	97.6	97.7	97.2
P. grimontii CFML 97-514^T (AF268029)	98.9	99.1	99.1	99.1	98.6	98.6	99.0	**99.9**	97.8	96.7	97.4	97.5	97.2
P. rhodesiae CIP 104664^T (AF064459)	98.7	98.8	98.9	99.0	98.4	98.4	98.8	**99.8**	97.8	96.7	97.4	97.4	97.2
P. marginalis ATCC 10844^T (AB021401)	98.4	98.5	98.6	98.7	98.1	98.1	98.5	**99.5**	97.6	96.5	97.1	97.3	96.8
P. moraviensis CCM 7280^T (AY970952)	97.0	97.0	97.2	97.0	97.7	97.7	96.9	97.4	**98.8**	**98.4**	**99.0**	99.0	96.8
P. koreensis Ps 3-14^T (AF468452)	97.2	97.2	97.2	97.1	98.1	98.0	97.0	97.5	**98.7**	**98.5**	**99.2**	**99.4**	96.6
P. jessenii CIP 105274^T (AF068259)	97.2	97.2	97.2	97.1	97.6	97.6	97.1	97.5	**98.8**	98.2	98.8	99.1	96.6
P. moorei RW10^T (AM293566)	96.7	96.6	96.8	96.6	97.0	97.0	96.5	96.9	97.9	97.1	97.8	98.5	95.9
P. vancouverensis DhA-51^T (AJ011507)	97.2	97.1	97.3	97.1	98.1	98.1	97.0	97.5	98.6	98.0	98.6	**99.8**	96.5
P. umsongensis Ps3-10^T (AF468450)	97.9	97.8	97.9	97.7	97.9	97.8	97.6	98.0	**98.9**	**98.5**	**99.1**	**99.2**	96.9
P. reinekei Mt-1^T (AM293565)	97.1	97.0	97.2	97.0	98.1	98.1	97.0	97.4	**98.8**	98.1	98.8	**99.4**	96.5
P. syringae LMG 1247^T (Z76669)	97.4	97.3	97.5	97.5	97.1	97.1	97.2	97.6	97.5	96.8	97.4	97.2	**98.1**
P. congelans DSM 14939^T (AJ492828)	97.5	97.5	97.7	97.7	97.1	97.1	97.0	97.9	97.5	96.8	97.4	97.2	**98.3**
P. fluorecsens Pf0-1 (CP000094)	96.9	96.8	96.9	96.7	97.7	97.7	96.7	97.1	**98.9**	**98.4**	**99.0**	**99.2**	96.5
P. fluorecsens Pf-5 (CP000076)	98.8	97.9	97.2	98.3	96.5	96.5	97.1	97.4	97.1	97.2	97.0	97.5	**99.7**
P. fluorecsens CHA0 (AJ278812)	97.1	97.0	97.2	97.4	96.7	96.7	97.1	97.5	97.1	96.4	97.0	96.7	**99.7**

Note: [1] The highest similarity values are shown in bold.

Low bootstrap values indicated that the branch of *P. salomonii* was unstable, as well as the positions of BIM B-143 and BIM B-147 within this branch.

Except for B-187, four studied strains were included into robust branches with type strains of *P. fluorescens* group. The common branch was formed by the type strain of *P. orientalis* and BIM B-100. BIM B-98 and BIM B-158 were stably linked to *P. cedrina* subsp. *cedrina* and *P. cedrina* subsp. *fulgida* type strains; the differences between BIM B-98–BIM B-158 and *P. cedrina* subsp. *cedrina*–*P. cedrina* subsp. *fulgida* exceeded the divergence of these subspecies.

BIM B-149 fell into the robust cluster formed by the strains of *P. grimontii, P. marginalis*, and *P. rhodesii*. Within this cluster it was most closely related to *P. rhodesii*, this fact being in contrast with the results of pairwise comparisons (Table 2). The differences between 16S rRNA gene sequences of bacteria from this group were as low as several nucleotides. This casts doubt upon the possibility of differentiation of these species.

BIM B-137 was grouped together with *P. veronii* type strain in the phylogenetic tree. A low bootstrap value indicated the instability of this sub-branch and high probability of changes in BIM B-137 position depending on the composition and length of the analyzed sequences.

None of the studied strains fell into the *P. syringae* phylogenetic group. A robust branch formed by strains of *P. fluorescens* Pf-5, *P. fluorescens* CHA0, and BIM B-222 was affiliated with this group. Judging by the low bootstrap values (less than 40), this branch did not belong to the *P. syringae* group and was not stably linked to it.

The last four bacteria (BIM B-86, BIM B-99, BIM B-156, BIM B-228) belonged to the phylogenetic group of *P. vancouverensis*. Only BIM B-228 was strongly related to the type strain of *P. vancouverensis*. Other bacteria were not linked to any species and formed several independent branches in this phylogenetic group.

These results confirm the high level of genetic heterogeneity of the collection strains of fluorescent pseudomonads. In spite of the fact that most of these bacteria were identified as *P. fluorescens* by phenotypic tests [9], comparative analysis of 16S rRNA gene sequences and phylogenetic analysis excluded the possibility of their affiliation within the same species and distributed them between relatively distant clusters of the genus *Pseudomonas* phylogenetic tree.

Thus, bacteria determined by using the classical identification scheme as *P. fluorescens* or *P. putida* were found to be taxonomically heterogeneous and in most cases not belonging to these species. The classical scheme of identification of bacteria of the *P. fluorescens* and *P. putida* groups is obviously archaic, and an alternative scheme has not been developed. For example, there is no reliable justification for the method of phenotypic differentiation of fluorescent pseudomonads in the corresponding chapters of the third edition of *The Prokaryotes* [14]. Hence, application of molecular techniques is obligatory in identification of fluorescent pseudomonads.

According to the results of 16S rRNA gene sequence comparative analysis, phylogenetic analysis, and genotyping results, only five bacteria could be identified with significant confidence: BIM B-187 belongs to *P. fluorescens*; BIM B-100, to *P. orientalis*; BIM B-98 and BIM B-158, to *P. cedrina*; and BIM B-228, to *P. vancouverensis*. The species affiliation of the other strains could be determined in the scope of this work only at the level of species group. More detailed investigation of these strains may result in the description of new species of the genus *Pseudomonas*.

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