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Unraveling the Extent of Diversity within the Order *Planctomycetales*

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The phylogenetic positions of 22 isolates that morphologically resemble members of the family *Planctomycetaceae* were determined by sequence analysis of genes coding for 16S rRNA. While nine and eight isolates could be assigned to the genera *Planctomyces* and *Pirellula*, respectively, three strains grouped near *Isosphaera pallida* and one strain was closely related to *Gemmata obscuriglobus*. No isolate was found to be related to a previously described species of any of the four genera at the species level. Morphological characters and sequence idiosyncrasies of genes coding for 16S rRNA of the isolates generally correlated with features described for the four genera to which the isolates could be assigned. One strain stands phylogenetically isolated and may be representative of a novel genus of the family. Comparison with environmental clone sequences representing planctomycetes in soil and water revealed that three of the novel isolates were related to one clone of soil origin, but no close relationships between clones and the other new strains were found. The study reveals that the biodiversity of planctomycetes is significantly greater than was previously determined.

During the 1920s, Gimesi (9) described planktonic, colony-forming microorganisms consisting of threadlike forms which bore spherical structures. These features were interpreted as fungal hyphae and exospores, respectively; therefore, this organism was described as a fungus, *Planctomyces bekefii*. A decade later, Henrici and Johnson (11), unaware of Gimesi's work, observed morphologically similar organisms which they interpreted as bacteria. The name *Blastocaulis sphaerica* was proposed for these stalked forms. The authors also found spore-forming, drop-shaped, colony-forming, budding bacteria lacking a stalk, which they believed to be identical to *Pasteuria ramosa* (29). These bacteria were later renamed *Blastobacter henricii* (62).

In the following decades, *P. bekefii*-like bacteria from middle and northern Europe (12, 18, 23, 30, 45, 55), Java (34), India, and Vietnam (19) were repeatedly reported. Besides *P. bekefii*, other budding bacteria were observed in diverse aquatic habitats such as eutrophic freshwater lakes (17, 23, 31, 32), fish ponds (18), brackish water (13, 17), aquarium water and marine sediments (17), and forest brook sediments (61). Stalkless organisms have been found in seawater (17) and freshwater lakes (15). A thermophilic form was found at 60°C in hot springs in Tiberias, Israel (21). Several *Planctomyces* species, such as *P. condensatus* (46), *P. stranskae* and *P. subulatus* (55), *P. ferrimorula* (56), *P. gracilis*, *P. crassus*, and *P. guttaeformis* (18), and *Blastocaulis kljasmensis* (62), were described. The identity of *P. bekefii* and *B. sphaerica* was described by Hirsch (14), who concluded that the name *Planctomyces* should have priority over *Blastocaulis*. The genus was restricted to "spherical, oblong or pear-shaped bacteria with long and slender stalks when in the mature state." Consequently, organisms that lacked a stalk were excluded from the genus *Planctomyces* and only *P. bekefii* Gimesi 1924 was included as the single species of this genus in the Approved Lists of Bacterial Names (44). Today, the genus contains three species described on the basis of pure culture studies, i.e., *P. maris* (1), *P. limnophilus* (16), and *P. brasiliensis* (37), while, in accordance with rule 18a of the International Code of Nomenclature of Bacteria (47), three species are described on the basis of descriptive type material, i.e., *P. bekefii*, *P. guttaeformis*, and *P. stranskae.*

It was not until 1973 that the isolation of a budding, aggregate-forming bacterium was reported. This stalkless bacterium had a morphology similar to that of *P. ramosa*, and although spores were not detected, it was described as the neotype of *P. ramosa* (51). Later, when a spore-forming bacterial parasite of daphniae (35) that resembled *P. ramosa* sensu Metchnikoff (29) was found, *P. ramosa* sensu Staley was renamed *Planctomyces staleyi* (53). Schlesner and Hirsch (39), however, argued that the genus *Planctomyces* should be restricted to stalked bacteria and proposed the genus *Pirella* (later renamed *Pirellula* [40]) for these stalkless organisms. Reports of other pure cultures of *Pirellula*-like bacteria are few: one strain was isolated from a freshwater pond (54), seven strains were isolated from the brackish water of the Baltic Sea (43, 49), and one strain was isolated from a contaminated tissue culture of the giant tiger prawn (8). Only one other species has been described, i.e., *Pirella marina* (36), now *Pirellula marina* (40).

Studies with pure cultures of strains of *Planctomyces*, *Pirellula*, and related genera revealed significant differences from all other known members of the domain *Bacteria*. These organisms lack a peptidoglycan layer in the cell wall but possess a proteinacious sacculus (22, 24), crateriform structures are located on the surface of the cells (41, 42), the 5S rRNA contains only 109 to 111 bases (2), and some species were shown to possess unlinked genes coding for 16S rRNA and 23S rRNA (16S rDNA and 23S rDNA, respectively) (26, 28). These striking differences led to the proposal of the order *Planctomycetales* (41), which phylogenetically represents a phylum in the domain *Bacteria* (50, 59). On the basis of morphological, chemotaxonomic, and phylogenetic evidence the order *Planctomycetales* and the family *Planctomycetaceae* were described for the genera *Planctomyces* and *Pirellula* and for an additional genus of budding bacteria with spherical to ovoid cells, *Gemmata* (7). Phylogenetic and phenotypic evidence also indicated that the taxon *Isosphaera pallida* (10), a multicellular, filamentous bacterium which is phototactic and moves by gliding, is also a member of this family.

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TABLE 1. List of strains, their origins, some phenotypic characters, and 16S rDNA accession numbers

Strain	Source of sample[a]	Morphology	Color	Motility	Accession number
1	Kiel Fjord	*Pirellula*-like	Pink	+	X81938
139	Kiel Fjord	*Pirellula*-like	Red	+	X81945
140	Kiel Fjord	*Pirellula*-like	Pink	−	X81939
158	Kiel Fjord	*Pirellula*-like	Pink	+	X81941
302	Chalk mine	*Pirellula*-like	Red	+	X81942
382	Aquarium	*Pirellula*-like	Red	+	X81943
384	Aquarium	*Pirellula*-like	Red	−	X81944
516	Algal basin, sewage treatment plant, Plön	Prosthecate cells	Red	−	X81940
678	Lake Fuhlensee	Spherical cells, 1-μm diameter	NC[b]	+	X81947
130	Kiel Fjord	*Planctomyces*-like	NC	+	X81952
269	Schrevenpark Pond	*Planctomyces*-like	Pink	+	X81953
638	Leakage water from compost heap	*Planctomyces*-like	NC	−	X81951
642	Leakage water from compost heap	Spherical cells, 1.0- to 1.5-μm diameter	NC	−	X81950
639	Leakage water from compost heap	*Planctomyces*-like	NC	−	X81950
658	Leakage water from compost heap	*Planctomyces*-like	NC	+	X81954
664	Leakage water from compost heap	*Planctomyces*-like	NC	−	X81955
668	Leakage water from compost heap	*Planctomyces*-like	NC	−	X81956
640	Leakage water from compost heap	Spherical cells, 2.0- to 2.5-μm diameter	NC	−	X81959
657	Leakage water from compost heap	Spherical cells, 2.0- to 2.5-μm diameter	Red	−	X81960
666	Leakage water from compost heap	Spherical cells, 2.0- to 2.5-μm diameter	Red	−	X81958
633	Leakage water from compost heap	*Gemmata*-like	Red	+	X81957
670	Leakage water from compost heap	Nearly rod shaped	NC	−	X81948

[a] All samples were from northern Germany.
[b] NC, not colored.

One can assume from the great phylogenetic depth of the genera *Planctomyces* and *Pirellula* and of the family *Planctomycetaceae* that the full range of biodiversity is not well represented by only nine described species. Recently, Schlesner (38) has developed different media which resulted in the enrichment and isolation of hundreds of budding bacteria that morphologically resembled strains of *Planctomyces* and *Pirellula*. In this communication, we present phylogenetic evidence that indeed many of the isolates represent hitherto unknown lineages within the confines of the family *Planctomycetaceae.*

MATERIALS AND METHODS

Enrichment and isolation. Strains were isolated from various habitats (Table 1). The enrichment and isolation procedures, habitats, and compositions of media were recently described (38).

16S rDNA sequence analysis. The extraction of genomic DNA and the amplification of 16S rDNA were performed as described previously (33). The PCR products were purified by using the Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) as described by the manufacturer. The *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) was used to directly sequence the PCR products, according to the protocol provided by the manufacturer. The sequence reactions were electrophoresed on the Applied Biosystems 373A DNA Sequencer.

Data analyses. The sequences were aligned against available sequences for members of the order *Planctomycetales*. Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (20). The least-squares distance method of De Soete (5) was used in the construction of phylogenetic dendrograms from distance matrices. Parsimony analysis, maximum likelihood, and neighbor joining were carried out by using the programs of the PHYLIP package (6). Bootstrapping analysis was used to determine the statistical significance of the branching patterns.

Nucleotide sequence accession numbers. Accession numbers of sequences are indicated in Table 1.

RESULTS AND DISCUSSION

16S rDNA sequence analysis was performed on 22 isolates from various aquatic habitats (Table 1) that on the basis of morphological features had been assigned to the order *Planctomycetales* (38). *P. brasiliensis*, which was not investigated in a previous phylogenetic study of members of the genera *Planctomyces*, *Pirellula*, *Gemmata*, and *Isosphaera* (25), was also included in this study. As comparison of published and novel 16S rDNA sequences indicated the presence of some potential ambiguities in the sequence of *P. staleyi* ATCC 27377^T, the sequence of this strain was reinvestigated. However, the uniqueness of several nucleotides in the sequence of this strain could be verified.

The almost complete 16S rDNA sequences, covering regions from position 99 through position 1435 (according to *Escherichia coli* nomenclature [3]) were determined. In contrast to the case for the vast majority of all bacterial species, for which the primary structure of 16S rDNA has been successfully determined by using described conserved sequencing primers (48, 57), the region from position 1 through position 372 of the 16S rDNA sequence of *P. brasiliensis* could not be analyzed. The reasons have not yet been investigated, but the inability may be caused by microheterogeneity in the multiple *rrn* operons reported to be present in other planctomycetes (26, 28), which may prevent the unambiguous determination of the sequence. In order to place *P. brasiliensis* within the radiation of planctomycete species, the analysis was restricted to the maximum information obtained for *P. brasiliensis*. As the branching pattern of the shorter sequences of described species was almost identical to that obtained by using complete sequences, the branching point of *P. brasiliensis* is indicated as a dotted line in the tree depicted in Fig. 1.

All isolates tentatively assigned to the family *Planctomycetaceae* on the basis of enrichment conditions and morphology were, indeed, found to be phylogenetically related to members of this family. The introduction of 22 novel sequences into the 16S rDNA database of only 6 sequences did not significantly change the topology of the previously published 16S rDNA tree (25). By using *Chlamydia psittaci*, a remotely related phylogenetic neighbor of the planctomycetes (58) as a root, two pairs of distantly related organisms can be detected, i.e., *Gemmata obscuriglobus* and *I. pallida* and members of *Planctomyces* and *Pirellula*, respectively. By using the same data set and maximum likelihood-neighbor joining analysis, the same branching order was recovered. Parsimony analysis, however,

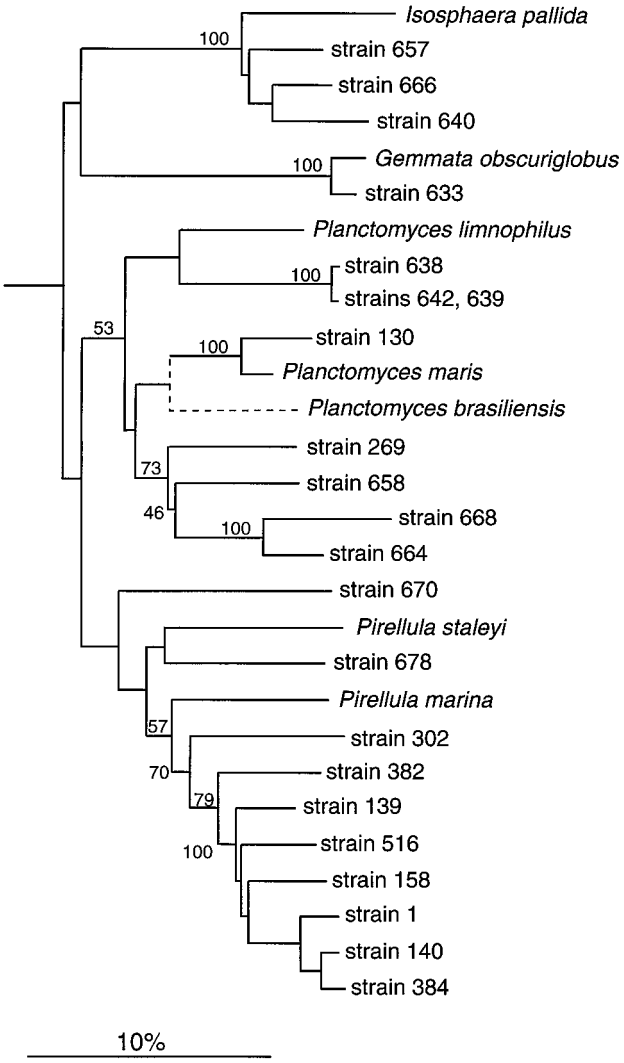

FIG. 1. 16S rDNA dendrogram indicating the phylogenetic relationships between described species of the family *Planctomycetaceae* (25) and isolates from various sites in northern Germany. Analysis was based on about 1,330 nucleotides. The root was determined by using the 16S rDNA sequence of *C. psittaci* as an outgroup reference (Table 2). The branching point of *P. brasiliensis* was determined on the basis of 1,050 nucleotides. The bar represents 10% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two organisms. Numbers refer to bootstrap values. Only values above 40% are shown.

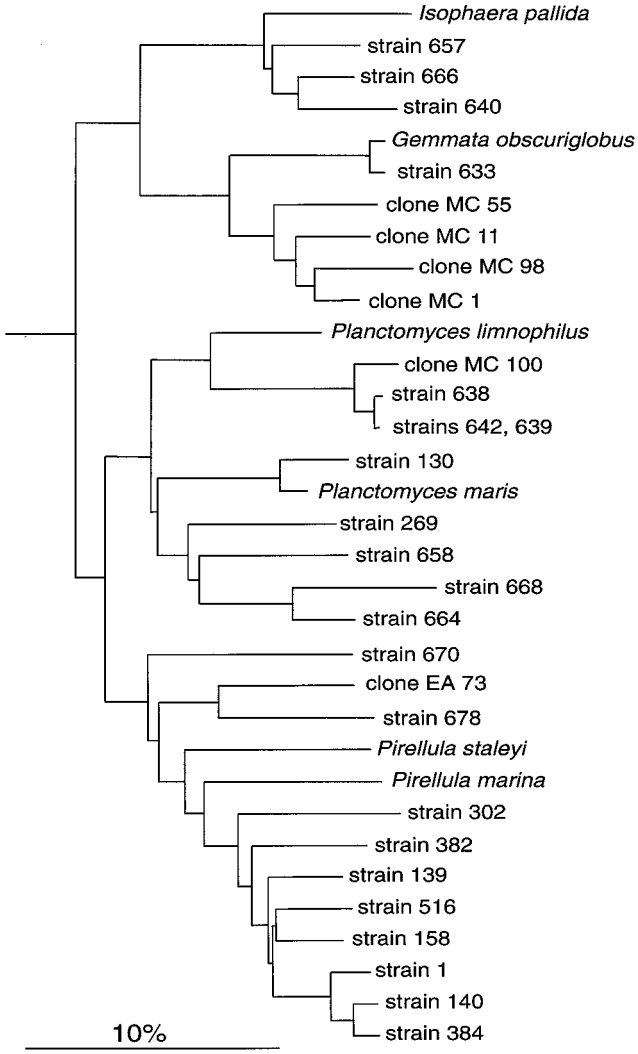

FIG. 2. 16S rDNA dendrogram indicating the phylogenetic relationships between described species of the family *Planctomycetaceae* (25), isolates from various sites in northern Germany, and sequences of clones generated from DNA extracted directly from the environment (MC, Mount Coot-tha, Brisbane, Australia [27]; EA, eastern Pacific Ocean [4]). Analysis was based on about 1,100 nucleotides. The root was determined by using 16S rDNA sequences of *C. psittaci* and a clone sequence, distantly related to planctomycete sequences, as outgroup references (similarity values not shown). The bar represents 10% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two organisms.

showed a closer relationship between *Pirellula marina* and *Pirellula staleyi* than was found by the other phylogenetic methods (data not shown). This is not surprising, since the bootstrap values for the branching points of these two species are low and therefore insignificant.

With the exception of strain 670, all isolates were found to be closely to moderately related to members of the four genera of *Planctomycetaceae*. The majority of the strains can be assigned to the genera *Pirellula* (nine strains; 85.0 to 89.1% similarity) and *Planctomyces* (eight strains; 83.8 to 95.9% similarity), while three strains were related to *I. pallida* (89.4 to 90.4% similarity) and one strain, strain 633, was related to *G. obscuriglobus* (97.1% similarity). Only a few isolates were found to be closely related to described species, i.e., strain 130 was closely related to *P. maris* and strain 633 was closely related to *G. obscuriglobus*. Several strains form clusters of higher relationships, found in *Pirellula* (isolates 1, 139, 140, 158, 384, and 516), *Planctomyces* (isolates 638, 639, and 642), and *Isosphaera* (isolates 640, 657, and 666). The relatedness of the isolates among themselves and to the described species as expressed by 16S rDNA similarity values is supported by high bootstrap values ranging between 53 and 100% and by certain sequence idiosyncrasies in variable 16S rDNA regions. With the exception of strain 139, all members of *Pirellula* have a long stem in the region from position 87 through position 97 (region defined in reference 60), consisting of 6 to 9 bp, while all other planctomycetes have only up to 5 bp. Strain 139 has a significantly longer stem consisting of 34 bp. Its closest relatives, strains 158 and 516 (94.1 and 93.3% similarity, respectively), have only a 7-bp stem. All the members of *Pirellula* and isolates 269, 658, 664, and 668 *Planctomyces* have a short stem of 3 bp

TABLE 2. Similarity matrix based on 16S rDNA sequences (Fig. 1)

Organism (isolate or species)[a]	% Similarity compared with organism no.:																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1 (1)																											
2 (140)	96.9																										
3 (384)	95.9	98.2																									
4 (516)	92.7	92.5	92.7																								
5 (158)	93.3	92.9	92.9	93.7																							
6 (302)	87.8	87.8	87.6	89.8	88.8																						
7 (382)	90.8	91.4	91.1	91.3	91.6	89.2																					
8 (139)	93.4	92.9	92.7	93.7	94.1	89.6	92.6																				
9 (P.st)	85.0	85.9	86.2	87.7	86.0	88.0	86.4	86.7																			
10 (P.ma)	87.1	87.4	87.7	89.1	87.1	87.8	88.9	89.2	87.5																		
11 (670)	83.2	83.9	84.1	85.0	84.7	83.4	84.4	85.4	86.5	84.6																	
12 (678)	85.5	85.3	85.2	86.3	86.4	86.2	86.0	87.1	87.2	86.5	85.2																
13 (Pl.li)	82.7	82.9	83.1	83.7	83.7	82.7	83.6	83.5	82.7	83.2	83.2	82.7															
14 (642)	81.8	81.8	81.5	81.7	82.2	81.3	82.8	82.7	82.5	82.3	82.3	81.6	89.1														
15 (638)	81.8	81.9	81.6	81.8	82.3	81.4	82.8	82.8	82.6	82.4	82.4	81.7	89.2	99.8													
16 (Pl.ma)	85.6	85.7	85.1	85.2	85.5	83.4	84.6	85.4	83.9	83.3	83.6	83.3	87.4	86.8	86.9												
17 (130)	84.5	84.1	83.4	83.6	83.7	82.0	82.8	83.5	82.1	81.7	81.9	81.5	86.5	85.8	85.9	95.9											
18 (269)	82.2	82.0	82.0	83.8	83.3	83.5	82.8	84.3	83.6	83.4	82.5	81.9	86.5	85.0	85.1	87.0	86.5										
19 (658)	82.8	82.9	82.4	82.3	82.7	83.5	83.7	83.9	83.1	82.8	82.1	82.0	85.6	84.4	84.4	86.8	86.0	90.2									
20 (664)	82.2	82.0	82.0	83.4	83.2	82.3	83.0	83.8	81.7	83.2	82.3	81.5	85.5	84.6	84.6	87.9	87.2	88.3	89.4								
21 (668)	79.6	79.8	79.6	80.0	79.7	79.2	80.4	80.2	79.4	80.4	79.9	78.9	83.8	82.8	82.8	85.4	84.6	86.4	87.1	92.7							
22 (G.ob)	78.6	78.9	78.7	80.5	80.3	79.3	80.6	81.4	80.8	81.1	81.2	80.8	79.9	78.7	78.8	80.5	79.0	80.3	79.5	80.0	77.7						
23 (633)	78.7	78.9	78.9	80.8	80.8	79.7	80.8	81.6	80.6	81.4	80.8	80.7	79.9	78.5	78.6	81.0	79.3	80.4	80.2	80.3	78.0	97.1					
24 (I.pa)	78.5	78.5	78.3	79.1	79.2	77.7	78.2	79.9	79.0	79.1	78.3	79.4	80.7	78.9	78.9	78.9	78.4	81.3	80.9	80.3	78.0	78.7	79.4				
25 (666)	80.3	80.4	80.5	81.4	81.6	79.5	80.9	81.7	80.2	80.7	80.8	81.1	82.5	82.4	82.5	82.0	80.8	82.0	81.3	81.1	77.7	79.7	80.1	90.4			
26 (640)	78.8	78.8	78.6	80.3	79.9	79.1	79.2	80.4	79.5	80.3	79.4	80.0	80.6	80.2	80.3	79.4	78.6	82.2	80.8	80.0	76.7	79.8	80.2	89.8	93.5		
27 (657)	81.0	80.1	80.2	81.5	81.8	80.6	81.0	82.6	80.8	81.3	81.4	81.9	81.4	81.8	81.9	80.9	79.4	82.1	81.4	81.0	78.0	80.7	81.0	89.4	92.2	92.7	
28 (C.ps)	72.3	73.2	73.0	73.3	73.8	71.8	73.3	73.7	72.6	73.4	72.6	72.6	73.1	72.6	72.7	74.4	72.7	73.6	74.3	72.3	71.3	72.3	72.5	71.2	72.4	71.2	72.6

[a] P.st, *Pirellula staleyi*; P.ma, *Pirellula marina*, Pl.li, *P. limnophilus*; Pl.ma, *P. maris*; G.ob, *G. obscuriglobus*; I.pa, *I. pallida*; C.ps, *C. psittaci.*

in the region from position 180 through position 194, while the other members of *Planctomyces*, as well as strains of *Gemmata* and *Isosphaera*, have a longer stem consisting of 7 to 12 bp. The insertion of about 20 bases in the region from position 1037 through position 1044, reported to be present in *G. obscuriglobus* (25), is also present in strain 633.

The allocation of the isolates to the described genera is by and large supported by their morphology (38, 52) (Table 1). Almost all members of *Pirellula* are ovoid to spherical and lack a stalk. Only strain 516 forms prosthecae. The majority of the strains allocated to *Planctomyces* show the typical genus-specific morphology, i.e., the presence of stalks. Interestingly, whereas strains 642 and 639 have identical 16S rDNA sequences, they differ in morphology. While a stalk could be detected in strain 639, cells of strain 642 appear spherical. However, as the presence of a short stalk can be verified only by electron microscopy, the presence of a stalk cannot be excluded by judging morphology only by light microscopy. Strain 633 exhibits the same morphology as its closest relative, *G. obscuriglobus*. The three isolates related to *Isosphaera* have spherical cells that are 2 μm in diameter and are thus significantly larger than the 1-μm-diameter cells of strain 642. Unbranched filaments observed in wild-type strains of *I. pallida* (52) were not detected in these novel strains. As the genus *Isosphaera* is defined by only a single strain, it is impossible to interpret the branching of the three isolates with respect to genus assignment. The phylogenetic depth of *I. pallida* and related strains, however, is much shallower than those of the genera *Planctomyces* and *Pirellula*. This may justify the tentative assignment of strains 640, 657, and 666 to the genus *Isosphaera*. Strain 670, standing phylogenetically isolated, is nearly rod shaped. As judged from the phylogenetic distance to the type strains of type species of planctomycete genera, this isolate may represent the nucleus of a new genus.

The distribution of motility and pigment color (Table 1) does not fully correlate with the phylogenetic relationships, but it is certainly useful for future species description.

In order to investigate the relatedness of 16S rDNA clone sequences, obtained from molecular ecological studies of Australian forest soil, abbreviated MC (27), and marine snow of the eastern Pacific Ocean, abbreviated EA (4), to the sequences obtained from the new isolates, the analysis had to be reduced to comparison of about 1,140 nucleotides (positions 99 through 1240). Environmental clone MC 18, representing organisms of a novel phylum distantly related to planctomycetes (27), and *C. psittaci* served as a root (Fig. 2 and Table 2). The positions of the clone sequences among isolates and described strains indicate that, except for one sequence, the clone sequences represent novel types of hitherto uncultured organisms. The four clones related to *G. obscuriglobus* branch off significantly lower than isolate 633 and may represent different species of a novel genus. Strain MC 100 is closely related to *Planctomyces* isolates 638, 639, and 642, while clone EA 73 is distantly related to the phylogenetically isolated *Pirellula* strain 678. It is interesting that the phylogenetic distances of clone sequences from each other and from the sequences of described species are similar to those separating the majority of isolates. Hence, as judged from these distances, one can assume that most of the isolates and most of the clones represent individual species.

As all attempts to isolate planctomycetes from these environments (from which the information about the presence of planctomycete DNA has been retrieved) have failed, information about morphology and other properties is lacking. The present study reveals that the application of novel enrichment and isolation procedures is a successful strategy for increasing our knowledge about the biodiversity of a group of bacteria previously represented by only a small number of species. The number of putative novel species of all four genera of the *Planctomycetaceae* isolated from a relatively small geographical region, i.e., northern Germany, exceed by almost threefold the number of validly described, cultured species previously isolated from four different continents. This study therefore demonstrates the significance and importance of applying novel and innovative enrichment and cultivation techniques in order to assess biodiversity and obtain better knowledge about biodiversity. Furthermore, this study demonstrates the suitability of 16S rDNA sequence analysis to unravel the degree of phylogenetic diversity within a new set of environmental isolates before full characterization is explored. The molecular ecological approach is another successful strategy for determining genetic diversity that would point toward the potential presence of novel types of organisms in any environment. This strategy would be even more convincing if accompanied by serious attempts to cultivate the relevant organisms.

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