Reclassification of four *Polynucleobacter necessarius* strains as representatives of *Polynucleobacter asymbioticus* comb. nov., *Polynucleobacter duraquae* sp. nov., *Polynucleobacter yangtzensis* sp. nov. and *Polynucleobacter sinensis* sp. nov., and emended description of *Polynucleobacter necessarius*

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Genome comparisons based on average nucleotide identity (ANI) values of four strains currently classified as *Polynucleobacter necessarius* subsp. *asymbioticus* resulted in ANI values of 75.7–78.4 %, suggesting that each of those strains represents a separate species. The species *P. necessarius* was proposed by Heckmann and Schmidt in 1987 to accommodate obligate endosymbionts of ciliates affiliated with the genus *Euplotes*. The required revision of this species is, however, hampered by the fact, that this species is based only on a description and lacks a type strain available as pure culture. Furthermore, the ciliate culture *Euplotes aediculatus* ATCC 30859, on which the description of the species was based, is no longer available. We found another *Euplotes aediculatus* culture (Ammermann) sharing the same origin with ATCC 30859 and proved the identity of the endosymbionts contained in the two cultures. A multilocus sequence comparison approach was used to estimate if the four strains currently classified as *Polynucleobacter necessarius* subsp. *asymbioticus* share ANI values with the endosymbiont in the Ammermann culture above or below the threshold for species demarcation. A significant correlation ($R^2$ 0.98, *P*<0.0001) between multilocus sequence similarity and ANI values of genome-sequenced strains enabled the prediction that it is highly unlikely that these four strains belong to the species *P. necessarius*. We propose reclassification of strains QLW-P1DMWA-1$^T$ (=DSM 18221$^T$=CIP 109841$^T$), MWH-MoK4$^T$ (=DSM 21495$^T$=CIP 110977$^T$), MWH-JaK3$^T$ (=DSM 21493$^T$=CIP 110976$^T$) and MWH-HuW1$^T$ (=DSM 21492$^T$=CIP 110978$^T$) as *Polynucleobacter asymbioticus* comb. nov., *Polynucleobacter duraquae* sp. nov., *Polynucleobacter yangtzensis* sp. nov. and *Polynucleobacter sinensis* sp. nov., respectively.

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Abbreviations: ANI, average nucleotide identity; ITS, intergenic transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the *rpoB*, *trpE*, *icdA*, *glnA*, *mdh*, *fbp*, *msbA* and *gyrA* genes of *Polynucleobacter necessarius* Ammermann are LN998990-LN998997; that for the 16S-23S ITS sequence is LN998998. The accession numbers for the genome sequences of *Polynucleobacter yangtzensis* MWH-JaK3$^T$ and *Polynucleobacter sinensis* MWH-HuW1$^T$ are LOJI00000000 and LOJJ00000000, respectively.

One supplementary table is available with the online Supplementary Material.
The genus *Polynucleobacter* and the species *Polynucleobacter necessarius* were proposed by K. Heckmann and H. J. Schmidt in 1987 for bacterial endosymbionts of freshwater ciliates affiliated with the genus *Euplotes*. Independent investigations demonstrated that these endosymbionts obligately rely on their host cells (Heckmann & Schmidt, 1987; Vannini et al., 2007) and thus represent obligate endosymbionts. This tight relationship between these bacteria and their hosts made it impossible to establish a pure culture representing the type of *P. necessarius* (Heckmann & Schmidt, 1987; Vannini et al., 2007). Thus, the genus *Polynucleobacter* and its type species *P. necessarius* are two of the few prokaryotic taxa not represented by a type strain. The type of the species *P. necessarius* is represented by a description of the endosymbionts contained in the *Euplotes aediculatus* stock 15 culture (=E24=ATCC 30859) (Heckmann & Schmidt, 1987). This mixed culture consists of the ciliate, algae serving as food for the ciliate, various free-living bacteria and endosymbionts of the genus *Polynucleobacter*. This culture is for several reasons not suitable for many purposes of comparative taxonomic research. The lack of a pure culture limits determination of new taxa by DNA–DNA reassociation experiments (DNA–DNA hybridization) or by chemotaxonomic traits, and phenotypical comparisons are restricted to morphological analyses. Owing to these limitations, the taxonomic classification of strains previously isolated as pure cultures from freshwater habitats (Hahn, 2003) was difficult (Hahn et al., 2009). These strains share 16S rRNA gene sequence similarities ≥99% with *P. necessarius* endosymbionts but dwell as free-living strains in the water column of freshwater lakes and ponds, thus differing from the endosymbionts profoundly in their lifestyle (Hahn et al., 2009, 2012b; Jezerova et al., 2010; Vannini et al., 2007; Wu & Hahn, 2006). Owing to the lack of pure genomic DNA of the endosymbiotic *P. necessarius*, it was not possible to rigorously test if the free-living and the endosymbiotic strains represent the same species. However, for pragmatic reasons, four free-living strains were preliminarily classified as *P. necessarius* strains due to the high 16S rRNA gene sequence similarity (≥99%) with endosymbiotic *P. necessarius*, tight phylogenetic clustering with endosymbionts in 16S rRNA gene trees and almost identical G+C values of their genomic DNA (Hahn et al., 2009). Because of the profound differences in lifestyle, separation of endosymbiotic and free-living *P. necessarius* strains into two subspecies, i.e. *P. necessarius* subsp. *necessarius* and *P. necessarius* subsp. *asymbioticus*, respectively, was proposed (Hahn et al., 2009). In this previous taxonomic study, four free-living strains representing the genus *Polynucleobacter* were characterized phenotypically and chemotaxonomically and classified as *P. necessarius* subsp. *asymbioticus* with strain QLW-P1DMA-1T as the type strain.

**Evaluation of the taxonomic position of strains previously classified as *P. necessarius* subsp. *asymbioticus***

Recently, genomic and ecological traits of two free-living strains previously described as *P. necessarius* subsp. *asymbioticus* (Hahn et al., 2009) were compared (Hahn et al., 2016). This investigation revealed an average nucleotide identity (ANI) value of 75.6% for complete genome sequences of strains QLW-P1DMWA-1T and MWH-MoK4T, which is far below the species demarcation threshold of 95–96% ANI suggested for prokaryotic species (Kim et al., 2014; Konstantinidis & Tiedje, 2005a; Konstantinidis et al., 2006).

In the study presented here, we evaluated the taxonomic position of all four free-living strains of the genus *Polynucleobacter* previously classified as *P. necessarius* subsp. *asymbioticus* (Hahn et al., 2009) by genome comparison. We determined the genome sequence of the remaining two strains (MWH-HuW1T and MWH-JaK3T) and completed the phenotypic characterization of strain MWH-MoK4T.

DNA used for genome sequencing was extracted from biomass grown in liquid NSY medium (Hahn et al., 2004) as described previously for another strain of the genus *Polynucleobacter* (Meinecke et al., 2012). Shotgun libraries were paired-end sequenced with an Illumina MiSeq instrument (Eurofins Genomics, Germany). *De novo* assembly of paired-end reads resulted for strains MWH-HuW1T and MWH-JaK3T in 19 and 42 contigs, respectively. In both cases, the genome size is about 2 Mbp (Table 1). Sequencing coverage was about 42× for strain MWH-HuW1T and about 17× for strain MWH-JaK3T. The draft genome sequences were annotated using the IMG/ER annotation pipeline (Markowitz et al., 2012) and deposited in DDBJ/EMBL/GenBank (Table 1).

The four strains previously classified as *P. necessarius* subsp. *asymbioticus*, i.e. strains QLW-P1DMWA-1T, MWH-MoK4T, MWH-HuW1T and MWH-JaK3T, shared >99% 16S rRNA gene sequence similarity and differed only marginally in genome size and G+C content of their DNA (Table 1). However, they differed in gene content (Table 2). Some of these gene content features were related to previously determined phenotypic traits. This included presence/absence of genes coding for utilization of urea as nitrogen source, catalase genes and genes encoding flagella proteins. In all three cases, gene content data are in partial conflict with previously reported phenotypic features of the strains. Growth on urea as sole nitrogen source was found previously in two strains (Hahn et al., 2009), but genes putatively encoding a urease and urea transporters were only detected in one of the four strains (Table 2). All four strains had been tested previously to be catalase positive, but genes putatively encoding this trait were only detected in two strains. All four strains had been tested by using soft agar plates as being non-motile. But on a closer look, strain MWH-MoK4T colonies may slowly spread on such plates, building swarms up to a diameter of 50 mm within 17 days, and this strain encoded the whole set of genes required for flagella synthesis. Interestingly, a recent microscopical investigation of this strain resulted in observation of a single cell spinning around its length axis. As previously reported, strain MWH-MoK4T also encodes a complete cluster of genes for synthesis of an anoxygenic photosynthesis system...
but cultures of the strain never showed any pigmentation revealing this trait (Hahn et al., 2016). It has to be considered that some of these differences in gene content and phenotype may simply result from lack of expression of the genes under the cultivation conditions used. In other cases, the discrepancies could result from annotation errors or insufficient phenotypic tests.

For evaluation of the taxonomic position of the four strains, we first tested which strains have to be considered to belong to the same species. Determination of pairwise ANI values by using the software JSpecies (Richter & Rosselló-Móra, 2009) resulted for all combinations in values in the range between 75.7 and 78.4 % (Table 3). Thus, all values are far below the threshold value of 95–96 % ANI suggested for the demarcation of prokaryotic species (Kim et al., 2014; Konstantinidis & Tiedje, 2005; Konstantinidis et al., 2006).

Next, we aimed to test if at least one of those four free-living strains may belong to the species *P. necessarius* represented by the endosymbionts of the genus *Polynucleobacter* in the mixed *Euplotes aediculatus* 'stock 15' culture (ATCC

Table 1. Major genome characteristics of the six taxa of the genus *Polynucleobacter* compared in this study

The upper four strains are currently classified as *P. necessarius* subsp. *asymbioticus* strains (Hahn et al., 2009), the obligate endosymbiont STIR1 is classified as *P. necessarius* subsp. *necessarius* (Boscaro et al., 2013), and strain 'beta proteobacterium' CB is lacking a sound classification (Hao et al., 2013) but clusters in 16S rRNA gene (Wang et al., 2009) and other phylogenetic trees (Figs 1 and 2) with *P. necessarius* strains.

| Strain            | Lifestyle | Genome size (Mbp) | DNA G+C content (mol%) | DDBJ/EMBL/GenBank accession number | IMG Genome ID | Reference                  |
|-------------------|-----------|-------------------|------------------------|------------------------------------|---------------|----------------------------|
| MWH-HuW1T (=DSM 21492T) | Free-living | 2.32              | 45.5                  | LOJJ00000000 2630969031            |               | This study                 |
| MWH-JaK3T (=DSM 21493T) | Free-living | 2.05              | 45.4                  | LOJJ00000000 2608642177            |               | This study                 |
| QLW-P1DMWA-1T (=DSM 18221T) | Free-living | 2.16              | 44.8                  | CP000655 640427129                |               | Meincke et al. (2012)     |
| MWH-MoK4T (=DSM 21495T) | Free-living | 2.03              | 45.2                  | CP007301 2505313000                |               | Hahn et al. (2016)         |
| 'beta proteobacterium' CB | Free-living | 2.05              | 46.1                  | CP004348 2565956558                |               | Hao et al. (2013)          |
| STIR1 (*Euplotes aediculatus*) | Endosymbiont | 1.56              | 45.6                  | CP001010 2503982034                |               | Boscaro et al. (2013)      |

Table 2. Differences in gene content between the four strains of the genus *Polynucleobacter* investigated taxonomically

The table represents an incomplete list of differences between genomes.

| Genes putatively encoding | QLW-P1DMWA-1T | MWH-JaK3T | MWH-HuW1T | MWH-MoK4T |
|---------------------------|---------------|-----------|-----------|-----------|
| Inorganic nutrients       |               |           |           |           |
| ABC-type Fe³⁺ transport system | –          | +         | +         | +         |
| feoAB genes (uptake of Fe²⁺) | +          | +         | +         | –         |
| ABC-type nitrate/nitrite/cyanate transporter | +          | +         | –         | –         |
| Nitrate reductase (assimilatory) | +          | +         | –         | –         |
| Nitrite reductase (assimilatory) | +          | +         | –         | –         |
| Cyanate lyase (releases NH₃ and CO₂ from cyanate) | +          | +         | –         | –         |
| Urease and ABC-type urease transporter | +          | –         | –         | –         |
| Oxidative phosphorylation/energy metabolism | | | | |
| Cytochrome bd-I terminal oxidase (CydAB) | +          | –         | +         | –         |
| Fumarate reductase | –          | +         | –         | +         |
| Carbon monoxide dehydrogenase | –          | +         | –         | +         |
| Acetate permease acT | +          | –         | –         | –         |
| Anoxygenic photosynthesis |               |           |           |           |
| Photosynthesis gene cluster | –          | –         | –         | +         |
| Motility                  |               |           |           |           |
| Flagella genes            | –          | –         | –         | +         |
| Oxidative stress          |               |           |           |           |
| Catalase                  | 2 genes      | 1 gene    | –         | –         |
| Other                     |               |           |           |           |
| Cellulose synthase operon protein C | +          | –         | –         | –         |
| Cellulose synthase catalytic subunit [UDP-forming] | +          | –         | –         | –         |
bionts, designated here as culture from MNHN and tested whether the endosymbionts of the genus contained the same endosymbionts of the genus established by D. Ammermann in 1969; thus, both should descend from the culture ATCC 30859 both descend from the culture placed previously by one of us in 2003. The culture finally received enabled us to better characterize the type of contained in this ciliate culture by resequencing of the 16S rRNA gene and by establishment of 16S–23S intergenic transcribed spacer (ITS) sequences (Vannini et al., 2007). Despite intensive independent efforts in two laboratories, we were not able to maintain the ciliate culture over longer periods of time. Recent searches for other sources of the culture ‘stock 15’ deposited by Heckmann Schmidt at ATCC in 1987 were unsuccessful. The Heckmann culture collection at the University of Münster, Germany, which was mentioned by Heckmann & Schmidt (1987) as a source of the culture, does not exist anymore, and culture requests to this university in June 2015 were unsuccessful.

The Euplotes aediculatus culture investigated by Heckmann & Schmidt (1987) had been isolated by Dieter Ammermann from ponds near Marseille, France in 1969 (Rao & Ammermann, 1970) and was later provided to Klaus Heckmann. The ciliate bearing the endosymbiotic bacteria was initially identified as Euplotes eurystomus (Rao & Ammermann, 1970) but later corrected as Euplotes aediculatus (Ammermann, 1971), which is highly similar to the former species. Importantly, the culture of Euplotes aediculatus established by D. Ammermann is still maintained at the Muséum National d’Histoire Naturelle (MNHN), Paris, France, and can be obtained from this institution if a material transfer agreement is signed. This culture and the culture ATCC 30859 both descend from the culture established by D. Ammermann in 1969; thus, both should contain the same endosymbionts of the genus Polynucleobacter. We obtained the Euplotes aediculatus Ammermann culture from MNHN and tested whether the endosymbionts, designated here as P. necessarius strain Ammermann, possessed the same 16S–23S ITS sequences as the endosymbionts contained in the previous ATCC culture. Primers used for amplification and sequencing are listed in Table S1 (available in the online Supplementary Material). The 16S–23S ITS sequence of P. necessarius strain Ammermann was found to be identical (Fig. 1) to the sequence obtained previously from culture ATCC 30859 (Vannini et al., 2007). BLAST searches with the ITS sequence obtained revealed that among the 224 ITS sequences of strains representing the genus Polynucleobacter currently deposited in DDBJ/EMBL/GenBank, only two organisms share an identical sequence with P. necessarius strain Ammermann. These are the endosymbions contained in ATCC 30859 and P. necessarius STIR1 contained in another culture of Euplotes aediculatus (Petroni et al., 2002; Vannini et al., 2007). All other ITS sequences of strains representing the genus Polynucleobacter share similarities in the range of 77–97%. This suggests that the endosymbions in the culture obtained from the MNHN are indeed identical with the type material of P. necessarius contained in ATCC 30859.

The most straightforward strategy for comparison of the four free-living strains with the endosymbions would have been genome sequencing of the endosymbions contained in the Euplotes aediculatus Ammermann culture. However, this would be a non-routine task because the endosymbions comprise only a very small fraction of the total DNA contained in the culture, and mass cultivation for yielding DNA amounts sufficient for sequencing of the endosymbiont genome with an acceptable coverage would be quite laborious. Instead of whole genome sequencing, we employed a multilocus sequencing approach with subsequent sequence comparisons and phylogenetic analyses. Assuming that P. necessarius strain Ammermann shares a high genome similarity with the completely sequenced P. necessarius STIR1 (Boscaro et al., 2013), we selected eight loci representing housekeeping genes scattered around the STIR1 genome and designed specific primers for partial amplification (Table S1). These primers enabled sequencing of the eight loci of P. necessarius strain Ammermann resulting in a total sequence length of 6087 bp. The concatenated sequence of the endosymbiont and the sequences extracted from the genomes of the four free-living strains possessed sequence similarities in the range of 81.8–88.4% (1108–704 nucleotide differences), while the sequences of P. necessarius strain Ammermann and STIR1 differed only in a single base (99.98% similarity). This single nucleotide polymorphism represents a synonymous substitution (T/C) at a third codon position of the gene (icdA) encoding the isocitrate dehydrogenase. The very high sequence similarity revealed at the eight loci is quite surprising since the sites of isolation of the two ciliates are located about 400 km apart in France.

### Table 3. Comparison of genomic similarity of taxa by pairwise calculation of ANI values

|                           | QIW-P1DMWA-1 T | MWH-MoK4 T | MWH-JaK3 T | MWH-HuW1 T |
|---------------------------|----------------|------------|------------|------------|
| STIR1 (Euplotes aediculatus) | 78.0           | 76.1       | 84.1       | 76.3       |
| QIW-P1DMWA-1 T (=DSM 18221 T) | 75.7           | 78.3       | 75.7       |            |
| MWH-MoK4 T (=DSM 21495 T)   |                | 76.4       | 78.4       |            |
| MWH-JaK3 T (=DSM 21493 T)   |                |            |            | 76.7       |
(Marseille) and Italy (River Stirone near Parma), and in addition, the establishment of the Ammermann culture took place about 30 years before the isolation of *Euplotes aediculatus* strain Ammermann. Results from analyses by the maximum-likelihood (ML) and maximum-parsimony (MP) methods are also indicated. Bootstrap values (percentage of replicates) above the threshold of ≥60% are shown for those nodes supported in at least one of the three methods; these bootstrap values are depicted in the order NJ/ML/MP. Bar, 0.05 substitutions per nucleotide position.

Next, we analysed whether sequence similarities of multilocus sequences correlate with whole-genome ANI values. Concatenated sequences homologous to the eight loci and genome sequences of six strains representing the genus *Polynucleobacter* (including 'beta proteobacterium' CB) and four strains representing the genus *Cupriavidus* (Fig. 2) were analysed (Fig. 3). This revealed a tight correlation of sequence similarity of the eight concatenated loci and ANI values ($R^2$ 0.98, $P$>0.0001), which enabled predictions of pairwise ANI values for the genomes of the four free-living strains investigated and *P. necessarius* strain Ammermann. The predicted values fell in the range of 77–85% ANI, which confirmed that none of the four free-living strains should be classified as a member of the species *P. necessarius*. Since the multilocus sequences of *P. necessarius* strain Ammermann and STIR1 were almost identical, it can be inferred that the genome sequence of STIR1 could be considered as a surrogate for the unavailable genomic DNA of *P. necessarius* 'stock 15' (ATCC 30859).

**Fig. 1.** Neighbour-joining (NJ) tree based on 16S–23S ITS sequences reconstructing the phylogenetic position of endosymbionts representing the genus *Polynucleobacter* contained in the culture of *Euplotes aediculatus* strain Ammermann. Results from analyses by the maximum-likelihood (ML) and maximum-parsimony (MP) methods are also indicated. Bootstrap values (percentage of replicates) above the threshold of ≥60% are shown for those nodes supported in at least one of the three methods; these bootstrap values are depicted in the order NJ/ML/MP. Bar, 0.05 substitutions per nucleotide position.

**Fig. 2.** Neighbour-joining tree calculated with concatenated multilocus sequences of eight loci representing housekeeping genes of bacteria of the genus *Polynucleobacter* (Table S1). Sequences of the endosymbiont *P. necessarius* strain Ammermann were obtained by using specific primers, whereas all other sequences were extracted from whole genome sequences. Strain 'beta proteobacterium' CB represents a strain affiliated with the genus *Polynucleobacter* whose genome has been sequenced previously (Hao *et al.*, 2013).
Polynucleobacter yangtzensis asymbioticus

We propose to establish four novel species, necessarius and transferred to novel species, respectively. Free-living strains should be excluded from the species.

Fig. 3. Correlation between ANI values obtained from whole genome comparisons and sequence similarities of the concatenated multilocus sequences (concatenated protein-coding loci listed in Table S1). The analysis included all taxa shown in Fig. 2 except P. necessarius strain Ammermann. The curve shown resulted from regression analysis with a three-parameter logarithmic equation. Note that the two data points with highest ANI and sequence similarity values resulted from comparisons of two strains representing the genus Cupriavidus, respectively.

be assumed that even the whole genomes are quite similar. This justifies an alternative opportunity for the estimation of the genome similarities between the four free-living strains, respectively, and P. necessarius strain Ammermann by direct comparison with the STIR1 genome (Table 3). As expected, these comparisons also resulted in ANI values below 85%.

Phylogenetic analyses of the concatenated multilocus sequences of bacteria of the genus Polynucleobacter and close relatives affiliated with the genus Cupriavidus resulted in separate clustering of members of these two genera (Fig. 2). Interestingly, the phylogenetic distances between taxa of the genus Polynucleobacter were quite large compared with distances obtained for strains representing distinct species of the genus Cupriavidus. The phylogenetic analyses performed further supports the separation of the four free-living strains representing the genus Polynucleobacter into four novel species.

Altogether, the ANI values obtained and the phylogenetic analysis of protein-encoding sequences enforce a revision of the current taxonomy of the species P. necessarius. All four free-living strains should be excluded from the species P. necessarius and transferred to novel species, respectively.

We propose to establish four novel species, Polynucleobacter asymbioticus comb. nov., Polynucleobacter duraquae sp. nov., Polynucleobacter yangtzensis sp. nov. and Polynucleobacter sinensis sp. nov., represented by the type strains QLW-P1DMWA-1T, MWH-MoK4T, MWH-JaK3T and MWH-HuW1T, respectively. These proposed type strains were described previously (Hahn et al., 2009), and data previously lacking for strain MWH-MoK4T are presented in Table 4.

P. necessarius, the four novel species and several undescribed taxa (Hahn et al., 2016) together form the so-called species complex PnecC within the genus Polynucleobacter (Hahn, 2003; Jezbera et al., 2011). This cryptic species complex is characterized by the presence of a diagnostic sequence (5′-GAGCCGGTGTTTCTTCCC-3′, Escherichia coli positions 445–463) in the 16S rRNA gene. This diagnostic sequence can be detected by using the PnecC-specific fluorescence in situ hybridization (FISH) probe PnecC-16S-445 (Hahn et al., 2005). However, assignment to a certain species within this cryptic species complex cannot be based solely on ribosomal sequences.

Characteristics for differentiation of strains affiliated with the genus Polynucleobacter from other members of the family Burkholderiaceae were published previously (Hahn et al., 2009). Differentiation of the four novel species of the genus Polynucleobacter from the previously described species Polynucleobacter cosmopolitanus, Polynucleobacter acidiphobus, Polynucleobacter difficilis and Polynucleobacter rarus is possible by using chemotaxonomic criteria. All four strains differ from strains representing P. rarus, P. difficilis and P. acidiphobus in the G+C content of DNA (Hahn et al., 2011a, b, 2012a) but cannot be discriminated by this feature from strains of P. cosmopolitanus (Hahn et al., 2010). Strains of the latter species are characterized by the presence of the fatty acid C12:0 3-OH, which was not detected in any other species of the genus Polynucleobacter characterized so far.

The discrimination of the four novel species from each other is possible by using the criteria presented in Table 5. The type strain of P. asymbioticus comb. nov. is the only strain able to assimilate L-aspartate, while the type strain of P. duraquae sp. nov. is the only strain which showed no assimilation of propionate. The type strain of P. sinensis sp. nov. is the sole strain able to assimilate both oxalacetate and L-glutamate, while P. yangtzensis sp. nov. is the only strain able to assimilate propionate but not both L-glutamate and L-aspartate. Furthermore, the type strain of P. asymbioticus comb. nov. differs from the three other type strains by the detection of the saturated fatty acid C20:0, while the type strain of P. duraquae sp. nov. differs from the others by the detection of the hydroxylated fatty acid C16:0 2-OH (Table 4). The type strain of P. sinensis sp. nov. differs from the other three type strains by the detection of the two fatty acids C17:0 and C15:0 10c; however, this trait may not be very reliable since both fatty acids contributed less than 1% to the total fatty acids.

Emended description of Polynucleobacter necessarius Heckmann & Schmidt 1987 emend. Hahn et al. 2009

Polynucleobacter necessarius (Pol.y.nucl.eo.bac.ter. Gr. adj. polys numerous; L. masc. n. nucleus nut, kernel; N.L. masc.
n. _bacter_ the equivalent of the Gr. neut. _n. bactron_ a rod; N. L. masc. _Polynucleobacter_ rod with many nucleoids; nec. L. adj. _necessarius_ indispensable, necessary).

This species belongs to the family _Burkholderiaceae_ and harbours obligatory endosymbiotic strains living in ciliates of the genus _Euplotes_. So far, endosymbiotic strains could not be cultured in pure culture (Vannini et al., 2007; Hahn et al., 2009).

Cells have elongated morphology with multiple nucleoid-like structures; penicillin-sensitive (Heckmann & Schmidt, 1987). Most probably descended from free-living strains of the genus _Polynucleobacter_ (Boscaro et al., 2013; Hahn et al., 2009; Vannini et al., 2007). Genome size is about 1.6 Mbp and its DNA G+C content is 44–46 mol%. The genome includes a large number of pseudogenes (Boscaro et al., 2013; Vannini et al., 2007), and the 16S rRNA gene sequence contains some unusual mutations not found so far in free-living strains of the genus _Polynucleobacter_ (Vannini et al., 2007). The type of the species _P. necessarius_ is represented by a description of the endosymbionts in the currently unavailable culture ATCC 30859. Endosymbionts of the genus _Polynucleobacter_ contained in the _Euplotes aediculatus_ Ammermann culture (Muséum National d’Histoire Naturelle, Paris, France) are considered to be identical with the endosymbionts in culture ATCC 30859. Endosymbionts of the genus _Polynucleobacter_ in the _Euplotes aediculatus_ STR1 culture were found to be highly similar genetically to the endosymbionts in the Ammermann culture. Therefore, this endosymbiont, characterized by a complete genome sequence, is also considered to be a member of the species _P. necessarius_. Gene and genome sequences characterizing these endosymbionts are available under the accession numbers AM397067, AM398078, LN998990-LN998998 and CP001010.

### Table 4. Major fatty acid contents of strains representing the four novel species of the genus _Polynucleobacter_

Data for strains QLW-P1DMWA-1T, MWH-JaK3T and MWH-HuW1T were taken from Hahn et al. (2009); however, fatty acid methyl esters had been prepared and measured under the same conditions as for strain MWH-MoK4T.

| Fatty acid | QLW-P1DMWA-1T | MWH-JaK3T | MWH-HuW1T | MWH-MoK4T |
|-----------|---------------|------------|------------|------------|
| C₁₂ : 0   | 3.4           | 3.7        | 5.5        | 3.8        |
| C₁₄ : 0   | 0.9           | 1.2        | 0.3        | 0.3        |
| C₁₅ : 0   | 0.3           | –          | 0.3        | –          |
| C₁₆ : 0   | 22.2          | 15.5       | 29.6       | 15.9       |
| C₁₇ : 0   | –             | –          | 0.5        | –          |
| C₁₈ : 0   | 1.2           | 0.5        | 2.4        | 0.5        |
| C₂₀ : 0   | 1.1           | –          | –          | –          |
| C₁₄ : ω₅c | –             | 0.6        | 0.2        | –          |
| C₁₅ : ω₆c | –             | –          | 0.6        | –          |
| C₁₆ : ω₅c | 0.9           | 0.4        | –          | 0.4        |
| C₁₆ : ω₇c | 41.3          | 35.6       | 45.0       | 38.6       |
| C₁₈ : ω₉c | –             | –          | 0.4        | 0.3        |
| C₁₈ : ω₇c | 12.9          | 20.4       | 1.1        | 19.8       |
| 11-Methyl C₁₈ : ω₇c | 3.1 | 8.1 | 1.1 | 4.2 |
| C₁₂ : 0 2-OH | 2.5 | 2.2 | 1.3 | 1.3 |
| C₁₆ : 0 2-OH | – | – | – | 1.8 |
| Summed feature 1 (including C₁₂ : 0 ALDE?) | 0.4 | 0.5 | 1.0 | – |
| Summed feature 2 (including C₁₄ : 0 3-OH) | 9.6 | 9.2 | 9.9 | 11.9 |
| Summed feature 7 (including C₁₉ : ω₆c) | 0.4 | 2.0 | 0.3 | – |

### Table 5. Characteristics for differentiation of the four proposed novel species of the genus _Polynucleobacter_

The method used for determination of assimilation capabilities was described previously (Hahn et al., 2009).

| Characteristic | QLW-P1DMWA-1T | MWH-JaK3T | MWH-HuW1T | MWH-MoK4T |
|---------------|---------------|------------|------------|------------|
| Assimilation of: |               |            |            |            |
| Propionic acid | +             | +          | +          | –          |
| Oxaloacetic acid | –             | +          | +          | +          |
| l-Glutamate | +             | –          | +          | –          |
| l-Aspartate | +             | –          | –          | –          |
Description of Polynucleobacter asymbioticus comb. nov.

Polynucleobacter asymbioticus (a.sym.bi.o’ti.cus. Gr. pref a not; N.L. masc. adj. symbioticus -a -um living together; N.L. masc. adj. asymbioticus not symbiotic).

Basonym: Polynucleobacter necessarius subsp. asymbioticus Hahn et al. 2009.

The description is based on phenotypical data of Hahn et al. (2009) and Hahn et al. (2012a), on chemotaxonomical data of Hahn et al. (2009), and on genomic data of Meicke et al. (2012), as well as data presented in Tables 1 and 2. Contains free-living strains of the genus Polynucleobacter dwelling in the water column of acidic or circum-neutral freshwater habitats. Cells are short rods, 0.7–1.2 µm in length and 0.4–0.5 µm in width. Chemo-organotrophic, aerobic and facultatively anaerobic. Can be cultivated on NSY, R2A, Luria–Bertani and peptone media. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs at up to 34 °C. Growth occurs with 0–0.4 % (w/v) NaCl. Weak assimilation is observed for some more substrates (Hahn et al., 2009). Does not assimilate glycolate, glyoxylate, propionate, malonate, oxalate, levulinic, D-mannose, D-galactose, L-fucose, D-sorbitol, L-glutamate, L-aspartate, L-alanine, L-serine, L-asparagine or citrate. Major cellular fatty acids are C16:1ω7c, C16:0, C18:1ω7c and summed feature 2 (including C14:0 3-OH). The sole 2-hydroxylated compound is C12:0 2-OH.

The type strain is MWH-MoK4T (DSM 21495T=CIP 110977T), which was isolated from alkaline Lake Mondsee (Hahn, 2003). The genome of the type strain is characterized by a size of 2.0 Mbp and a DNA G+C content of 45.2 mol%. Gene and genome sequences characterizing the type strain are available under the accession numbers CP0007501 and AJ550654.

Description of Polynucleobacter yangtzensis sp. nov.

Polynucleobacter yangtzensis (yang.tzen’sis. N.L. masc. adj. yangtzensis of or belonging to the Yangtze River, the river from where the type strain was isolated).

The description is based on phenotypical data of Hahn (2003) and Hahn et al. (2009), on chemotaxonomical data of Hahn et al. (2009) and on genomic data presented in this study (Table 2). Contains free-living strains of the genus Polynucleobacter dwelling in the water column of freshwater systems. Cells are short rods, 0.5–1.5 µm in length and 0.3–0.5 µm in width. Chemo-organotrophic, aerobic and facultatively anaerobic. Can be cultivated on NSY, nutrient broth, peptone, soytone, yeast extract, tryptic soy media, Standard agar and R2A media. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs at up to 35 °C. Growth occurs with 0–0.3 % (w/v) NaCl. Weak assimilation is observed for some more substrates (Hahn et al., 2009). Does not assimilate glycolate, glyoxylate, oxalate, levulinic, D-mannose, D-glucose, D-galactose, D-sorbitol, L-glutamate, L-aspartate, L-alanine, L-serine, L-asparagine or citrate. Major cellular fatty acids are C16:1ω7c, C18:1ω7c, C16:0 and summed feature 2 (including C14:0 3-OH and iso-C16:0 1). The sole 2-hydroxylated compound is C12:0 2-OH.

The type strain is QLW-P1DMWA-1T (=DSM 18221T=CIP 109841T), which was isolated from a small acidic freshwater pond located in the Austrian Alps at an altitude of 1300 m (Hahn et al., 2005). The genome of the type strain is characterized by a size of 2.2 Mbp and a DNA G+C content of 44.8 mol%. Strains affiliated with this species are characterized by the diagnostic sequence 5‘-ACTAAGGCGATCTAA TGATTTGTTA-3‘ in the 16S–23S rRNA (Jezbera et al., 2011). Gene and genome sequences characterizing the type strain are available under the accession numbers CP000655 and AJ879783.

Description of Polynucleobacter duraque sp. nov.

Polynucleobacter duraque (dur.a’qua. L. adj. dura -a -um hard; L. fem. n. aqua water; N.L. gen. fem. n. duraque from/of hard water, i.e. water with higher concentrations of dissolved limestone).

The description is based on phenotypical data of Hahn (2003) and Hahn et al. (2009), on chemotaxonomical data of Hahn et al. (2009), data presented in Table 4 and genomic data presented in Table 1. Contains free-living strains of the genus Polynucleobacter dwelling in the water column of alkaline or circum-neutral freshwater systems. Never found in acidic waters (Hahn et al., 2016; Jezbera et al., 2011). Cells are curved rods, 0.9–2.9 µm in length and 0.4–0.5 µm in width. Chemo-organotrophic and aerobic; anaerobic growth was not observed. The type strain encodes a gene cluster for anoxygenic photosynthesis but expression of a photosynthesis system has not been observed so far. Encodes genes for synthesis of flagella but motility is usually not observed. Can be cultivated on NSY, peptone, yeast extract, R2A and Luria–Bertani media. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs at up to 30 °C. Growth occurs with 0–0.3 % (w/v) NaCl. Assimilates acetate, pyruvate, oxaloacetate, succinate, fumarate and L-cysteine. Weak assimilation is observed for some more substrates (Hahn et al., 2009). Does not assimilate glycolate, glyoxylate, propionate, malonate, oxalate, levulinic, D-mannose, D-galactose, L-fucose, D-sorbitol, L-glutamate, L-aspartate, L-alanine, L-serine, L-asparagine or citrate. Major cellular fatty acids are C16:1ω7c, C18:1ω7c, C16:0 and summed feature 2 (including C14:0 3-OH). The sole 2-hydroxylated compound is C12:0 2-OH.
The type strain is MWH-JaK³T (=DSM 21493T=CIP 110976T), which was isolated from the Yangtze River (Hahn, 2003). The species epithet does not indicate that the distribution of the taxon is restricted to a certain geographic area. The genome of the type strain is characterized by a size of 2.0 Mbp and a DNA G+C content of 45.4 mol%. Gene and genome sequences characterizing the type strain are available under the accession numbers LOJ10000000 (version LOJ10000000) and AJ550657.

Description of Polynucleobacter sinensis sp. nov.

Polynucleobacter sinensis (sin'en'sis, N.L. fem. adj. sinensis pertaining to China, the country where the bacterium was isolated).

The description is based on phenotypical data of Hahn et al. (2009), on chemotaxonomical data of Hahn et al. (2009) and on genomic data presented in this study (Table 2). Contains free-living strains of the genus Polynucleobacter dwelling in the water column of freshwater systems. Cells are curved rods, 0.6–1.4 μm in length and 0.4–0.5 μm in width. Chemo-organotrophic and aerobic; anaerobic growth is not observed. Shows good growth on NSY and R2A media. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs at up to 35 °C. Growth occurs with 0–0.5% (w/v) NaCl. Assimilates acetate, propionate, malonate, malate, pyruvate, oxaloacetate, succinate, fumarate and l-glutamate. Weak assimilation is observed for D-glucose, D-lactose, D-mannose, D-mannitol, D-xylose, D-fructose, L-fucose, D-sorbitol, L-aspartate, L-alanine, l-serine, L-asparagine or citrate. Major cellular fatty acids are C₁₆:1ω7c, C₁₆:0 and summed feature 2 (including C₁₄:0 3-OH and C₁₆:0 (iso I)). The sole 2-hydroxylated compound is C₁₂:0 2-OH.

The type strain is MWH-HuWI³T (=DSM 21492T=CIP 110978T), which was isolated from a slightly alkaline, artificial pond (31° 20' 14.81" N 120° 34' 34.20" E) at Tiger Hill (Huqiu) located in Suzhou, PR China (Hahn, 2003). The species epithet does not indicate that the distribution of the taxon is restricted to a certain geographic area. The genome of the type strain is characterized by a size of 2.3 Mbp and a DNA G+C content of 45.5 mol%. Gene and genome sequences characterizing the type strain are available under the accession numbers LOJ10000000 (version LOJ10000000) and AJ550666.

Acknowledgements

We thank Anne Aubusson Fleury for helping us to find the Euplotes aediculatus culture originally established by Dieter Ammermann. We are also grateful to Marc Dellinger (MNHN) for providing information on the history of the culture obtained from the Muséum National d’Histoire Naturelle, as well as for advice regarding maintenance of this culture. We thank Gabriele Pöller for carrying out the fatty acid measurements. This study was supported by the Austrian Science Fund (FWF) project 1482–B09 (Ecological diversification in Polynucleobacter), and the European Science Foundation (ESF) project FREDI.

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