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Endosymbiosis In Statu Nascendi: Close Phylogenetic Relationship Between Obligately Endosymbiotic and Obligately Free-Living *Polynucleobacter* Strains (*Betaproteobacteria*)

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Running title: Complete niche separation in *Polynucleobacter* strains

Summary

Bacterial strains affiliated to the phylogenetically shallow subcluster C (PnecC) of the *Polynucleobacter* cluster, which is characterized by a minimal 16S rRNA gene sequence similarity of approx. 98.5%, have been reported to occur as obligate endosymbionts of ciliates (*Euplotes* spp.), as well as to occur as free-living cells in the pelagic zone of freshwater habitats. We investigated if these two groups of closely related bacteria represent strains fundamentally differing in lifestyle, or if they simply represent different stages of a facultative endosymbiotic lifestyle. The phylogenetic analysis of 16S rRNA gene and 16S-23S ITS sequences of five endosymbiont strains from two different *Euplotes* species and 40 pure culture strains demonstrated host-species-specific clustering of the endosymbiont sequences within the PnecC subcluster. The sequences of the endosymbionts showed characteristics indicating an obligate endosymbiotic lifestyle. Cultivation experiments revealed fundamental differences in physiological adaptations, and determination of the genome sizes indicated a slight size reduction in endosymbiotic strains. We conclude that the two groups of PnecC bacteria represent obligately free-living and obligately endosymbiotic strains, respectively, and do not represent different stages of the same complex lifecycle. These closely related strains occupy completely separated ecological niches. To our best knowledge, this is the closest phylogenetic relationship between obligate endosymbionts and obligately free-living bacteria ever revealed.

Introduction. In 1975, Heckmann described a bacterial endosymbiont of the freshwater ciliate *Euplotes aediculatus* (Hypotrichia) and named it "omikron" (Heckmann, 1975). These bacterial endosymbionts were found to be individually contained in cytoplasmic vesicles, to which ribosomes are often attached. The omikron endosymbiont is characterized by the presence of usually 3-9 nucleoid-like structures (Heckmann and Schmidt, 1987). So far, omikron-like endosymbionts were reported from nine *Euplotes* species, all but one of these species inhabit freshwater systems (Görtz, 2002; Petroni et al, 2002).

Heckmann demonstrated that *Euplotes aediculatus* cured from their endosymbionts by antibiotic treatment were able to perform 1-2 more cell divisions but died 15-20 days after the last division (Heckmann, 1975; Heckmann and Schmidt, 1987). Treatment of aposymbiotic ciliates with ciliate homogenate containing intact omikron-like endosymbionts or by direct injection of symbiont-containing cytoplasm resulted in both, the reestablishment of the endosymbiont population, and the recovery of the temporarily aposymbiotic ciliates (Heckmann, 1975; Fujishima and Heckmann, 1984). Repeated attempts to cultivate the endosymbionts in pure culture failed completely (Heckmann, 1975). Based on these observations it was concluded that omikron is an obligately endosymbiotic bacterium, which is essential for its ciliate host (Heckmann, 1975; Heckmann et al., 1983; Heckmann and Schmidt, 1987). Meanwhile, the dependence of at least eight *Euplotes* species on omikron-like endosymbionts was demonstrated (Heckmann et al., 1983; Vannini et al., 2005).

In 1987, Heckmann & Schmidt described the omikron endosymbiont of *Euplotes aediculatus* as *Polynucleobacter necessarius* (Heckmann and Schmidt, 1987). This species is one of a few validly described bacterial species lacking a pure culture of its type strain. In 1996, Springer and colleagues determined the 16S rRNA gene sequence of the type strain, and verified the affiliation of the sequence to the endosymbiont by fluorescence in situ hybridization (FISH) with a *P. necessarius*-specific probe (Springer et al., 1996).

From 1996 on, cloning and sequencing of PCR-amplified fragments of bacterial 16S rRNA genes from surface freshwater habitats yielded more than 100 sequences related to the sequence of the endosymbiont *P. necessarius* (e.g., Bahr et al., 1996; Hiorns et al., 1997; Semenova and Kuznedelov, 1998, Simek et al., 2001). The monophyletic cluster formed by these environmental sequences and the *P. necessarius* sequence was first designated ACK-1 cluster (Hiorns et al., 1997), and later, after the discovery of the affiliation of a valid species with this cluster, it was renamed as *Polynucleobacter necessarius* cluster (Zwart et al., 2002). With one exception (Pedersen et al., 1996) the more than 100 retrieved environmental sequences affiliated with the *P. necessarius* cluster were obtained from surface freshwater habitats. The *Polynucleobacter* cluster contains four monophyletic, species-like (minimal 16S rRNA gene sequence similarity >97%) subclusters (Hahn, 2003). The 16S rRNA sequences of five previously investigated endosymbionts of the two ciliate species *Euplotes aediculatus* (Springer et al., 1996; Vannini, et al., 2005) and *E. harpa* (Vannini, et al., 2005) are affiliated with subcluster C (PnecC) of the *Polynucleobacter* cluster. This subcluster also contains cultivated strains isolated by the filtration acclimatization method (Hahn et al., 2004) from the pelagic zone of diverse freshwater habitats (Hahn, 2003). The highest 16S rRNA gene sequence similarity between a PnecC strain isolated from freshwater and the *P. necessarius* type strain is 99.5%.

Recently, it was demonstrated by fluorescence in situ hybridization (FISH) with subcluster-specific probes that strains affiliated to subcluster PnecC or other *Polynucleobacter* subclusters occur with high abundances (up to 10^6 cells ml^{-1}) as free-living cells in the plankton of freshwater habitats (Hahn et al., 2005; Wu and Hahn, 2006a; Wu and Hahn, 2006b). Furthermore, it was demonstrated that every ribotype (16S-23S ITS genotype) detected by a cultivation-independent method in a particular habitat could be cultivated by the acclimatization method (Hahn et al., 2005). On the other hand, all previous attempts to

obtain pure cultures of the *P. necessarius* endosymbionts of *E. aediculatus* by standard microbiological methods failed (Heckmann, 1975).

At first glance, it seems that the strains affiliated to the species-like subcluster PnecC fall into two groups, i.e., a group of free-living strains, and a group of endosymbiotic strains. However, it is not known if these two groups differ fundamentally in lifestyle, i.e., if the one group represents obligately free-living and the other group represents obligately endosymbiotic strains, or if both groups simply represent different stages of a facultative endosymbiotic lifestyle. Previously, it was suggested that the isolated strains and the endosymbionts differ completely in lifestyle (Hahn, 2003). This suggestion was based on morphological differences between the two groups of bacteria (differences in cell shape and size, and lack of the 3-9 nucleoid-like structures in strains isolated from freshwater). Meanwhile, pure cultures of new PnecC strains were established, which share with the endosymbiotic stages some of their characteristic morphological features (Hahn, unpublished data), however, the presence of the common traits (i.e., elongated cells and nucleoide-like structures) was always restricted to minorities of the cells in the pure cultures. This observed morphological plasticity of some cultivated PnecC strains does not support the previous conclusions on differences in lifestyles of free-living and endosymbiotic stages.

In the study presented here, we investigated if the endosymbiotic and the free-living stages of PnecC bacteria really differ fundamentally in lifestyle. We examined if (i) free-living and endosymbiotic stages differ in culturability, (ii) compared the 16S rRNA gene and ITS sequence of 40 cultivated PnecC strains and 5 endosymbiotic strains, (iii) searched for endosymbiotic genotypes in the pelagic of freshwater habitats with specific fluorescence in situ hybridization (FISH) probes, (iv) searched for potential genome reduction in endosymbiotic strains by comparison of genome sizes of endosymbiotic and free-living stages, and (v) investigated if the free-living and the endosymbiotic stages differ in the

presence of deleterious mutations in their ribosomal sequences. The obtained results support the hypothesis of fundamental differences in the lifestyles of free-living and endosymbiotic PnecC bacteria. Thus, the species-like subcluster PnecC contains closely related obligate endosymbionts and obligate free-living strains. Consequently, the subcluster PnecC contains at least three groups of strains (two groups of host-specific endosymbionts, and the free-living strains) occupying completely separated ecological niches. To our best knowledge, the PnecC bacteria represent the closest phylogenetic relationship between obligate endosymbionts and obligately free-living bacteria ever revealed, and the *Polynucleobacter-Euplotes* system most likely represents the evolutionary youngest obligate endosymbiosis discovered so far.

Results

Analysis of ribosomal sequence. The 16S rRNA gene sequences and 16S-23S ITS sequences of 45 strains affiliated with the species-like subcluster PnecC of the *Polynucleobacter* cluster were analyzed. The 45 strains included 40 pure culture strains isolated from freshwater habitats (subsequently termed free-living strains), and five endosymbiotic strains hosted by the ciliates *Euplotes aediculatus* (two strains) and *E. harpa* (three strains) (Table 1). Phylogenetic analysis as well as analysis by BLAST indicated that the set of 16S rRNA sequences of the 40 pure culture strains included those *Polynucleobacter* sequences closest related to the endosymbiont sequences, i.e. public data bases did not contain any environmental *Polynucleobacter* sequence more similar to the endosymbiont sequences as the analyzed sequences. The aligned sequence set consisted of 2022 positions (1505 positions belonged to the partial 16S rRNA gene sequences, and 517 positions to the ITS sequences). The 16S rRNA gene sequence newly obtained from the type strain of *Polynucleobacter necessarius* (harbored by *E. aediculatus* strain ATCC 30859) differed in 4

positions from the previously determined sequence (acc. number X93019, (Springer et al.,
146 1996)). None of the 4 positions in the new sequence were in conflict with the secondary structure model of the 16S rRNA provided by the ARB software package, therefore, further
148 analysis was based on the new sequence.

The 16S rRNA gene and ITS sequences of the two endosymbionts of *E. aediculatus*
150 strains ATCC 30859 and STIR1, which were obtained from sites located more than 400 km apart from each other (Fig. 1), were identical over the entire length of 2002 nucleotides (0
152 polymorphic sites). The three sequences obtained from the endosymbionts of the three *E. harpa* strains (isolated from habitats located up to 3000 km apart) possessed one polymorphic
154 position in the 16S rRNA gene, and three polymorphic positions in the ITS. In contrast, sequences of the 40 free-living PnecC strains contained 106 polymorphic sites, and sequences
156 of strains obtained from the same habitat possessed 2 to 60 polymorphic sites (9 habitats; 2 to 6 isolates per habitat; in average 25.2 polymorphic sites in sequences of strains from the same
158 habitat). The highest number of polymorphic sites in sympatric strains was found in six isolates obtained from Lake Mekkojärvi (Finland).

160 Phylogenetic trees based on 16S rRNA gene or ITS sequences, respectively, of the 45 PnecC strains and several strains affiliated with other *Polynucleobacter* subclusters were
162 calculated by three methods. In all trees the endosymbiont sequences clustered within the radiation of subcluster PnecC (Fig. 2 and 3). The endosymbiont sequences obtained from *E.*
164 *aediculatus* and *E. harpa* hosts never appeared in a common cluster separated from all sequences of free-living strains, but appeared as two separate host-specific clusters within the
166 PnecC subcluster. The sequences of the two host-specific groups of endosymbionts showed for both phylogenetic markers the highest divergence from other members of this subcluster
168 (Fig. 2 and 3). Besides the higher number of mutations in all five ribosomal sequences of endosymbionts, the three *E. harpa* endosymbionts possessed in comparison to other members

of the *Polynucleobacter* cluster a significantly higher number of base pair mismatches in helices (according to the secondary structure model provided by the software package ARB) (Fig. 4). Interestingly, this trait is not shared by the two endosymbionts of *E. aediculatus*.

The 16S rRNA gene sequences of the endosymbiotic strains possessed host species-specific signatures. For instance, both endosymbionts of *E. aediculatus* contain an insertion of a guanine nucleotide between positions 193 and 194 (*E. coli* numbering), and all three endosymbionts of *E. harpa* possessed the same signature of seven single-nucleotide substitutions in stem regions, resulting in mismatches in base-pairing. These characteristics were neither found in any of the >100 *Polynucleobacter* strains so far isolated from freshwater habitats, nor in one of the >100 environmental *Polynucleobacter* sequences deposited in the public databases.

The 16S rRNA gene sequences of the endosymbiotic *Polynucleobacter* bacteria and the free-living *Polynucleobacter* strains differ only in a few positions. Such small sequence dissimilarities have so far not been reported for obligate endosymbionts and their closest free-living relatives (Table 2).

Genome size and G+C content of *Polynucleobacter* bacteria. The genome size of three endosymbiotic and eleven free-living *Polynucleobacter* strains were determined by pulsed field gel electrophoresis (PFGE). The endosymbiotic strains possessed genome sizes of 1.7 and 1.8 Mb, while the eight free-living strains affiliated with subcluster PnecC possessed significantly larger genome sizes (t-test, $P<0.001$) ranging from 2.1 to 2.5 Mb (Fig. 5). Genome sequencing of the freshwater isolate QLW-P1DMWA-1 confirmed the genome size determined for this strain by PFGE (2.2 Mb in both cases). On the other hand, the three investigated free-living strains affiliated with subcluster PnecD possessed genome sizes, which did not differ significantly from the genome size of the endosymbiotic PnecC strains

(t-test, P>0.1). Though the genus *Polynucleobacter* is affiliated with the family
196 *Burkholderiaceae* (*Burkholderiales*, *Betaproteobacteria*), *Polynucleobacter* strains studied here differ significantly in genome size (Fig. 5B) and G+C content (Fig. 6) from all other
198 *Burkholderiaceae* strains investigated so far.

200 **Cultivation experiments with two endosymbiotic strains.** Attempts to cultivate two endosymbiotic *Polynucleobacter* strains by using the filtration-acclimatization and the
202 dilution-acclimatization methods did not result in pure cultures of the strains. In the case of the filtration-acclimatization method, the failure of the experiment was most likely a result of
204 the inability of the endosymbiotic strains to pass through 0.2 µm filters. Microscopical investigations of 0.2 µm-filtrates of the endosymbiont-containing ciliate homogenate did not
206 result in the detection of endosymbionts. Nevertheless, samples were further processed by the acclimatization method, but no *Polynucleobacter* cultures were finally obtained.

208 In the experiments performed with the dilution-acclimatization method, the presence of intact endosymbiont cells in the homogenates used as inoculum was verified microscopically.
210 Diluted and undiluted homogenates were acclimatized to higher substrate concentrations, and finally screened for the presence of PnecC bacteria by FISH. In order to increase the chances
212 of successful cultivation, different initial media were used in the acclimatization processes. In some experiments, acclimatization to higher substrate concentrations was substituted by
214 cultivation in sterile ciliate homogenate instead. None of these cultivation experiments did result in any cultures of *Polynucleobacter* strains. Several established cultures, however,
216 showed growth of non-*Polynucleobacter* strains, which obviously originated from free-living strains, which were present in large numbers in the ciliate cultures and as minor fractions in
218 the ciliate homogenate used for the cultivation experiments. The growth of these bacteria

demonstrated, however, that the homogenates used for the cultivation experiments contained viable bacteria.

Investigation of ciliate cultures by FISH. The sequence of probe Omi10 published by Springer et al. (Springer et al., 1996) did not match with the 16S rRNA sequence of the *P. necessarius* type strain (ATCC30859) published by the same authors, but matched perfectly with the sequence obtained from the type strain in our study, as well as the sequence of *P. necessarius* strain STIR1 (Vannini et al., 2005). Therefore, we used in our study probe Omi10, as well as the new probe Omi10-mod, which was adjusted to the 16S rRNA sequence of *P. necessarius* ATCC30859 published by Springer et al. Both probes did not match (one or two mismatches) with any of the more than one hundred 16S rRNA sequences of all free-living strains, or with the sequences of the three endosymbiotic *Polynucleobacter* strains found in *E. harpa* (Vannini et al., 2005). Hybridization conditions appropriate for the discrimination of the *P. necessarius* endosymbionts of *E. aediculatus* from all other members of subcluster PnecC were established for both probes. The appropriate formamide concentration for the original probe Omi10 was found to be much higher than the concentration for probe Omi10-mod (65% versus 35%). This may indicate that the original probe fits perfectly to the sequence of the 16S rRNA gene, however, probe Omi10-mod also allowed the discrimination of *P. necessarius* endosymbionts from the other PnecC strains. FISH performed on *Euplotes* spp. cells picked one by one by a micropipette and fixed on a glass slide resulted in the detection of many *Polynucleobacter* symbionts inside of the ciliate cells, but never resulted in the detection of *Polynucleobacter*-like bacteria outside the ciliate cells. Different results, i.e. the detection of cells targeted by the endosymbiont-specific probe outside of ciliates were observed when ciliates were filtered onto 0.2µm pore size membrane filters prior to hybridization. The majority of probe-positive cells were located nearby to

ciliate cells (< 100 µm), which indicates that this detection of endosymbiont genotypes outside of ciliate cells was an artifact resulting from the damaging of ciliate cells during the filtration process.

Since the two Omi probes did not match the sequences of the *E. harpa* endosymbionts, probe Poly_862 was used for the investigation of the *E. harpa* cultures. Again, probe-positive bacteria were detected exclusively inside of the host cells when ciliate cells have been handled carefully.

Investigation of environmental samples by FISH. Samples from the pelagic zone of five lakes and one pond were investigated by FISH (Table 3). The PnecC-specific probe PnecC-16S-445 detected free-living bacteria in samples from all six investigated freshwater habitats. All cells detected possessed small cell sizes typical for bacterioplankton cells. Probe-positive cells attached to particles or organisms, as well as cells located in any protist cell were not detected. Abundances of probe-positive cells ranged from 3.7×10^3 to 6.8×10^5 cells ml^{-1}. By contrast, probe Omi10-mod, which is specific for endosymbionts of *E. aediculatus*, did not detect any cells in the investigated environmental samples.

Experiments on enrichment and cultivation of *Euplotes* species from habitats containing free-living *Polynucleobacter* bacteria. Enrichments were established from samples of three lakes and one pond. These three lakes are known to contain populations of free-living *Polynucleobacter* bacteria (Table 3). *Euplotes* species were not observed in any of the established enrichment cultures, however, ciliates belonging to other genera could be enriched and subsequently isolated (data not shown).

Discussion

The independent phylogenetic analysis of 16S rRNA genes and 16S-23S ITS sequences placed *Polynucleobacter* strains isolated from the pelagic of freshwater habitats, the endosymbionts of the freshwater ciliate *E. aediculatus*, and the endosymbionts of the brackish water ciliate *E. harpa* in the species-like subcluster PnecC of the *Polynucleobacter* cluster (Fig.2 and 3). This cluster represents in terms of genome size (Fig. 5) and G+C content of ITS sequences (Fig. 6B) a homogenous group well separated from other members of the family *Burkholderiaceae*. The minimal divergence of 16S rRNA genes (0.5%) of strains isolated from freshwater habitats and endosymbionts of *Euplotes* spp. suggested a close phylogenetic relationship of these organisms. Fundamental differences in lifestyle of such closely related bacteria have not been reported so far.

For two reasons, it is highly likely that the isolated strains existed as free-living, planktonic bacteria prior to isolation. First, the investigation of a free-living population of PnecC strains in the water column of a freshwater habitat by cultivation-independent and cultivation methods resulted in the detection and cultivation of the same ribosomal genotypes (Hahn et al., 2005). This demonstrated that the environmental sequences and the isolates obtained originated from a free-living population. Second, it is known, that the potential hosts of endosymbiotic *Polynucleobacter* strains, i.e. *Euplotes* spp., are usually absent from the pelagic of freshwater habitats (Foissner et al., 1991; Foissner et al., 1999). These ciliates usually inhabit interstitial, benthic sites rich in organic material (i.e., mud and detritus), and only in exceptional cases *Euplotes* spp. were reported from the pelagic of aquatic systems (Foissner et al., 1999). The few observations of planktonic *Euplotes* spp. were always restricted to anoxic parts of the pelagic zone, but all isolated *Polynucleobacter* strains were obtained from oxic water bodies. By contrast, the five strains termed above “endosymbiotic strains” undoubtedly represent endosymbionts of the two *Euplotes* species. This was

repeatedly demonstrated by FISH (Springer et al., 1996; Vannini et al., 2005), and electron microscopy (Vannini et al., 2005).

The occurrence of strains affiliated with the phylogenetically narrow subcluster PnecC as free-living and endosymbiotic stages could either be explained by a facultative endosymbiotic lifestyle of all members of the subcluster, or the members of the subcluster could fundamentally differ in lifestyle, i.e. could represent obligate endosymbionts and obligately free-living strains. The results presented here strongly argue against a facultative endosymbiotic lifestyle of the PnecC bacteria. The endosymbiotic strains demonstrated several characteristics of obligate symbionts. The analysis of the ribosomal sequences of the endosymbionts revealed a tight relationship between host species and endosymbiont genotypes (Fig. 2 and 3). This host-specific linkage stands in strong contrast to the large geographical distance between the sampling sites of the endosymbionts (Fig. 1), and to the significant ribosomal gene diversity within subcluster PnecC (Fig. 3). Both clearly indicate an obligate symbiotic relationship between the hosts and the endosymbionts. The cultivation experiments indicated pronounced physiological differences between the free-living stages and the endosymbiotic stages. The inability of the endosymbiotic strains to grow in media supporting growth of free-living strains could be a result of a genetic adaptation to the environmental conditions in the cytoplasm of the host cells. This may include the loss of metabolic capabilities necessary for the survival as free-living strains, or for growth in the provided media. The lack of culturability is one indicator for obligate endosymbiosis known from numerous bacterial endosymbionts, e.g., *Buchnera* (Tamas et al., 2002), *Wigglesworthia* (Aksoy, 1995), *Blochmannia* (Sauer et al., 2000), and is interpreted as a loss of unneeded metabolic pathways (Moran, 2002). The lack of culturability of the *Polynucleobacter* endosymbionts, and the previously reported dependence of host cells on their endosymbionts (Heckmann, 1975; Vannini et al., 2005) indicate an obligate interdependence of

endosymbionts and hosts, which is a typical characteristic of obligate endosymbiosis (Zientz et al., 2004). The loss of genes encoding dispensable metabolic function also results in reduced genome sizes of obligate endosymbiotic bacteria (Moran, 2002). In several cases, their genomes are reduced to ~20% of the genome size of the closest related free-living relatives (Moran et al., 2003). The majority of obligate endosymbionts investigated so far possess genome sizes of < 1.5 Mb, while the majority of free-living bacteria possess genome sizes of 2 to 10 Mb (Moran, 2002). In contrast to all obligate endosymbionts investigated so far (e.g. Shigenobu et al., 2000, Tamas et al., 2002) the genome size of the endosymbiotic *Polynucleobacter* differed much less significantly from that of the closest free-living relatives (Fig. 5). Another characteristic of the genomes of obligate endosymbionts is the accumulation of mildly deleterious mutations (Lambert and Moran, 1998). Our analysis indicated destabilizing base substitutions in the 16S rRNA genes of *E. harpa* endosymbionts but not in the *E. aediculatus* and the free-living strains (Fig. 4). Furthermore, the two groups of endosymbionts showed each for both investigated phylogenetic markers, i.e. the 16S rRNA genes and the ITS sequences, the highest divergence from other members of subcluster PnecC. This may indicate elevated rates of sequence evolution compared to free-living relatives, which was repeatedly demonstrated for other obligate endosymbionts (Aksoy, 1995; Herbeck et al, 2003).

The lack of pronounced genome reduction in the *Polynucleobacter* endosymbionts, as well as the lack of an increased number of mismatches in helix regions of the 16S rRNA of *E. aediculatus* endosymbionts is in agreement with the proposed close phylogenetic relationship between the endosymbionts and their closest free-living relatives. This close relationship suggests that the time point of divergence of the endosymbiotic and free-living *Polynucleobacter* bacteria was much more recent than in the case of other obligate

endosymbionts. In the case of *Buchnera* and their closest free-living relatives a divergence period of >100 million years was estimated (Moran et al., 2003).

In contrast to the endosymbiotic strains, an endosymbiotic lifestyle was not evident for the free-living strains. Large free-living *Polynucleobacter* populations were observed in the pelagic zone of habitats from which potential hosts, i.e. *Euplotes* spp., could not be enriched. Even if *Euplotes* spp. have been present in these habitats, their population sizes must have been several orders of magnitude smaller than the size of the free-living *Polynucleobacter* populations. This has to be expected due to the known restriction of these ciliates to the benthic zone, and due to further restrictions caused by a partially low habitat suitability (Foissner et al., 1991; Foissner et al., 1999) of the benthic zone in these lakes. The resulting imbalance between the population size of free-living *Polynucleobacter* bacteria and potential hosts makes a symbiotic relationship between these organisms unlikely. Furthermore, the free-living and the endosymbiotic *Polynucleobacter* strains differ from known facultative symbionts (e.g. *Vibrio fischeri*) and known facultative parasites of protists (e.g. *Legionella* spp.) in the lack of motility. Due to this lack *Polynucleobacter* strains should be in principal unable to chemotactically find potential hosts.

Conclusions. The presented results indicate that the phylogenetically shallow subcluster PnecC harbors both, obligately free-living strains and obligate endosymbionts of at least two ciliate species. Consequently, this subcluster harbors at least three groups of organisms occupying completely separated ecological niches. Niche differentiation is well documented for more distantly related bacteria. For instance, Gray et al. (2004) demonstrated niche differentiation in sympatric *Achromatium* spp., which are separated by 16S rRNA gene sequence similarities of 91.5%, and Schauer et al. (2005) demonstrated complete niche separation in two groups of filamentous bacteria both affiliated with the SOL cluster but separated by 16S rRNA gene sequence similarities of 92.1%. The finding of niche separation

in *Polynucleobacter* bacteria, as well as the ecological differentiation in *Brevundimonas alba* strains sharing identical 16S rRNA gene sequences (Jaspers & Overmann, 2004) highlights the ecological significance of microdiversity.

To our best knowledge, the phylogenetic relationship between the obligately endosymbiotic and the obligately free-living *Polynucleobacter* bacteria is the closest currently known. The closest free-living relatives of all other obligate endosymbionts investigated so far are more distantly related (Table 2). We did not find any example of an obligate endosymbiont, which also shares a 16S rRNA gene sequence similarity >99% with its closest free-living relative. Further investigations on the evolutionary adaptations of *Polynucleobacter* endosymbionts and their hosts may provide exciting insights into processes taking place during the early evolutionary phase of endosymbiosis.

Experimental procedures

Microbial strains, culture conditions, and media. The *Polynucleobacter* endosymbionts of five ciliate strains of the genus *Euplotes* (Hypotrichia) were investigated (Table 1). Three of the strains belong to the species *Euplotes harpa*, and two strains belong to *Euplotes aediculatus*. The strain *E. aediculatus* ATCC 30859 harbors the type strain of *Polynucleobacter necessarius*. The three *E. harpa* strains were cultured in artificial seawater at low salinity (5‰) at 20°C. The *E. aediculatus* strain STIR1 was cultured in sterile spring water enriched with boiled rice grains at 20°C, and *E. aediculatus* strain ATCC 30859 was cultured in sterile WC medium (Guillard, 1975) at 22°C. *Euplotes aediculatus* strain ATCC 30859 was fed with the algae *Cryptomonas* sp. SAG26.80, and the other strains were fed with the green algae *Dunaliella tertiolecta*. All ciliate cultures were non-axenic. The cultures were kept in the light, either under permanent illumination (*E. aediculatus* ATCC 30859), or under a light dark cycle of 12:12 hours (other strains).

Determination of 16S rRNA gene sequences and 16S-23S internal transcribed spacer (ITS1) sequences. For the determination of ribosomal sequences of endosymbiotic *Polynucleobacter* strains, the host ciliate cells were separated from free-living bacteria also present in the ciliate cultures by filtration onto a 15µm pore size membrane filter, washed with sterile medium, and finally resuspended in sterile medium. Ciliates were pelleted by centrifugation at 200g for ten minutes. DNA was extracted from the obtained biomass. The forward *Polynucleobacter*-specific primer 16S-Poly-F467 (Vannini et al., 2005) and a reverse primer binding at position 457 (*E. coli* numbering) of the 23S rRNA gene (Ludwig et al., 1992) were used for the amplification of ITS sequences of the endosymbionts from three *E. harpa* strains and from *E. aediculatus* STIR1. For the amplification of the 16S rRNA gene and the 16S-23S internal transcribed spacer (ITS) of the endosymbiont of *E. aediculatus* strain E24 (ATCC 30859) the forward primer 19F (Hahn et al., 2005) and the PnecC-specific reverse primer PnecCr-5 (Hahn et al., 2005) were used. Amplification of 16S rRNA genes and ITS sequences of free-living strains were performed as described previously (Hahn, 2003; Hahn et al., 2005).

Comparative sequence analysis. Sequences were aligned with the autoalignment tool provided by the software package ARB (Ludwig et al., 2004), and alignment errors were corrected manually. Sequence polymorphisms were analyzed with the software DnaSP 4.1 (Rozas et al., 2003). Phylogenetic trees were calculated by using several methods. The DNAPARS program was used for maximum parsimony analysis (Felsenstein, 1989), with a bootstrap analysis of 1,000 pseudoreplicates. A maximum likelihood analysis (Felsenstein, 1981; Olsen et al., 1994) was performed by the FastDNAml tool, and a neighbor joining analysis (Saitou and Nei, 1987) was performed by the DNADIST tool with Kimura correction (Kimura, 1980), as well as by the software Mega2 (Kumar et al., 2001).

Determination of genome size. The genome size of three endosymbiotic strains, eight free-living PnecC, and three free-living PnecD strains was determined by pulsed field gel electrophoresis (PFGE). *Polynucleobacter*-harboring ciliates and free-living *Polynucleobacter* cells were harvested and washed in sterilized artificial sea-water and phosphate-buffered saline, respectively. Pellets were immobilized in agarose plugs and treated as described by Strous et al. (2006). PFGE was then performed in a contour-clamped homogeneus electric field system (CHEF-DR III; Bio-Rad) at 11°C, using 1x TAE pH 8 and 1% Seakem Gold Agarose (Cambrex). Gels were run by three resolution blocks, differing for pulse, angle and time. Fragment length was estimated by comparison with standard size ladders (*Saccharomices cerevisiae* and *Hansenula wingei* PFGE Markers; Bio-Rad).

Cultivation experiments with endosymbionts. Cultivation experiments with endosymbionts of *E. aediculatus* strain STIR1 and of *E. harpa* strain FSP1.4 were performed by using the acclimatization method (Hahn et al., 2004), which was successfully used for the cultivation of environmental isolates (Hahn, 2003; Hahn et al., 2005). For the separation of endosymbionts from free-living bacteria present in the non-axenic ciliate cultures, the ciliate cells were filtered through a 100 μm pore-size filter, harvested onto 15 μm pore-size filters, and resuspended in sterile IBM medium (Hahn et al., 2004). Forty ml of these suspensions were harvested by centrifugation at 200g (10 min) and concentrated in 4 ml IBM medium. The endosymbionts were released from the hosts by destroying the ciliates by centrifugation at 6,000g (15 min) and subsequent vortexing. Presence of released endosymbionts in the homogenate was verified by staining with DAPI (4',6'-diamino-2-phenylindole) and epifluorescence microscopical inspection. Furthermore, the presence of free-living bacteria in the homogenate was tested by DAPI-staining and hybridization with the probe PnecC-16S-445 (see below), which is specific for the *Polynucleobacter* cluster but did not hybridize with free-living bacteria present in the ciliate cultures. The homogenates containing the highly

enriched endosymbionts were used for two different cultivation experiments. (i) Samples were processed according to the standard protocol of the filtration-acclimatization method (FAM (Hahn et al., 2004)) (Table 4). This method employs filtration through 0.2µm filters for the separation of target organisms from organisms able to overgrow the target organisms. (ii) Samples were processed by the dilution acclimatization method (DAM (Hahn et al., 2005)). This method employs a dilution step instead of the filtration step. Briefly, samples of 100 µl of the homogenate were diluted into six different concentrations (1:1 to 1:100000). One hundred µl of the dilutions were used for the inoculation of wells of sterile cell culture plates (24-wells) containing 500 µl of the starting medium. Four different approaches were tried in the DAM experiments: (i) stepwise acclimatization to NSY 3 gl^{-1} (Hahn et al., 2004) using IBM as a starting medium; (ii) acclimatization to NSY 3 gl^{-1} using ciliate homogenate filtered through a 0.2 µm pore-size membrane as a starting medium, and stepwise addition of NSY 3 gl^{-1}; (iii) cultivation attempts without any acclimatization (i.e. inoculation of NSY 3gl^{-1} with dilutions of the enriched endosymbionts), and (iv) cultivation in ciliate homogenate filtered through 0.2 µm pore-size filters without increasing the substrate concentration (i.e. no addition of medium). For experiments (i) and (ii) the standard acclimatization protocol described by Hahn et al. (2004) was followed (i.e. stepwise addition of increasing amounts of dissolved organic material). In experiments (ii) and (iv) controls receiving no inoculum were used for testing sterility of the 0.2µm-filtered ciliate homogenate. Screening of the wells for the growth of bacteria was done by dropping 10 µl samples from each well onto NSY agar plates, or in the case of experiment iv onto 0.2 µm-filtered ciliate homogenate solidified with agar (15 g/l). Samples of colonies grown on plates were tested for the presence of *Polynucleobacter* bacteria by FISH using the probes Omi10-mod and PnecC-16S-445 (see below).

Experiments on the isolation of *Euplotes* strains from freshwater habitats. The benthic zone of Lake Krottensee, Lake Wolfgangsee, Lake Egelsee (M2), and an unnamed pond were sampled at sites appropriate for freshwater *Euplotes* species. Samples were microscopically screened for the presence of *Euplotes* spp. immediately after sampling, as well as after several days of incubation. Aliquots of the samples were also enriched with prey organisms appropriate for *Euplotes* (i.e. *Dunaliella tertiolecta*, *Phaeodactylum* sp., *Enterobacter aerogenes*) and daily screened for the presence of *Euplotes* spp. over a twenty-days period.

Analysis of ciliate cultures and environmental samples by FISH. Samples taken from ciliate cultures and from the pelagic of six freshwater habitats located in the Austrian Salzkammergut area (near Salzburg) were investigated by fluorescence in situ hybridisation (FISH). Twenty ml samples were fixed with paraformaldehyde solution (2% final concentration) and filtered onto 0.2µm polycarbonate membrane filters as described elsewhere (Hahn et al., 2005). Whole-cell *in situ* hybridizations of sections of the filters were performed as described previously (Hahn et al., 2005). In parallel, ciliate cells were also processed in a different way: they were picked one by one, together with their culture medium, by a glass micropipet and then fixed on microscope slides. Slides were then processed for FISH as described elsewhere (Petroni et al., 2003). Four different Cy3-labelled oligonucleotide probes were used for the hybridization of samples. (i) Probe PnecC-16S-445 (Hahn et al., 2005), which is specific for the entire subcluster C (PnecC) of the *Polynucleobacter* cluster. (ii) Probe Omi10 (Springer et al., 1996), which is specific for the endosymbiotic *Polynucleobacter necessarius*, (iii) probe Omi10-mod (5'-TTT CCC CCC TTC AGG GCG T-3', the underlined nucleotide is not contained in the probe Omi10), which is a slightly modified version of Omi10, and (iv) probe Poly_862 (Vannini et al., 2005), which is specific for the entire *Polynucleobacter* cluster. Probe Omi10 is matching the 16S rRNA sequence of *P. necessarius* strain ATCC30859 (sequence obtained in this study), and

the sequence of *P. necessarius* STIR1 (Vannini et al., 2005), while probe Omi10-mod is matching the previously published sequence of *P. necessarius* ATCC30859 (Springer et al., 1996). Probe Omi10-mod was applied with 35% formamid concentration in the hybridization buffer, probe Omi10 with 65%, and probe Poly_862 with 0% formamid (Vannini et al., 2005).

Nucleotide sequence accession numbers. The obtained 16S-23S ITS sequences of endosymbiotic and free-living PnecC strains were submitted to the DDBJ/EMBL/GenBank databases under accession numbers XXXXXXXX to XXXXXXXX. The 16S rRNA gene sequences of these strains have been deposited previously.

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Table 1. Investigated endosymbionts and their hosts.

Endosymbiont	Host	Host strain	Origin		Deposition	Reference
			Habitat	Site		
Polynucleobacter sp.	*Euplotes harpa*	FSP1.4	Brackish water	Pisa, Italy	-	Vannini et al., 2005
Polynucleobacter sp.	*Euplotes harpa*	BOD2	Brackish water	Boderne, Denmark	-	Petroni et al., 2002
Polynucleobacter sp.	*Euplotes harpa*	FC1	Brackish water	Taranto, Italy	-	Vannini et al., 2005
Polynucleobacter necessarius	*Euplotes aediculatus*	STIR1	Freshwater	Parma, Italy	-	Petroni et al., 2002
Polynucleobacter necessarius	*Euplotes aediculatus*	ATCC 30859[a]	Freshwater	Marseille, France	ATCC[b]	Heckmann & Schmidt, 198

[a] ATCC 30859 is identical with clone 15 of *E. aediculatus* (Heckmann & Schmidt, 1987)
[b] ATCC: Amarican Type Culture Collection

Table 2. 16S rRNA similarities between obligate endosymbionts and the known closest related free-living species or strains. Closest relatives of the endosymbionts were found by BLAST. Sequence similarities were determined after alignment of downloaded sequences by using the software program ARB.

Obligate Endosymbiont	Accession Number	Host	Closest free-living species/strain	Phylogenetic distance (16S rRNA similarity)	Reference (for endosymbiont)
Endosymbiont of *Ixodes scapularis*	AB001518	Tick (*Ixodes scapularis*)	*Flexibacter tractuosus*	83.1 %	Kurtti et al., 1996
Endosymbiont of *Acanthamoeba* sp. UWC8	AF069963	Amoebae (*Acanthamoeba* sp. UWC8)	*Ehrlichia ruminantium* [b]	84.3 %	Fritsche et al., 1999
Candidatus Tremblaya princeps	AF476095	Mealybug (*Pseudococcus viburni*)	*Burkholderia cepacia*	85.6 %	Thao et al., 2002
Caedibacter caryophilus	X71837	Ciliate (*Paramecium caudatum*)	*Azorhizobium caulinodans*	85.7 %	Springer et al., 1993
Wigglesworthia glossinidia	L37342	Tsetse flies (*Glossina tachinoides*)	*Pectobacterium carotovorum*	88.0 %	Aksoy, 1995
Candidatus Blochmannia rufipes	X92552	Ant (*Camponotus rufipes*)	*Serratia marcescens*	88.5 %	Sauer et al., 2000
Candidatus Procabacter acanthamoebae	AF177427	Amoebae (*Acanthamoeba* sp. UWC12)	*Azovibrio restrictus*	88.7 %	Horn et al., 2002
Buchnera aphidicola str. APS	NC_002528	Aphid (*Acyrthosiphon pisum*)	*Shigella flexneri*	89.3 %	Shigenobu et al., 2000
Parachlamydia acanthamoebae	Y07556	Amoebae (*Acanthamoeba castellanii*)	*Waddlia chondrophila* [b]	89.4 %	Everett et al., 1999
Buchnera aphidicola str. Sg	NC_004061	Aphid (*Schizaphis graminum*)	*Escherichia coli*	89.4 %	Tamas et al., 2002
Trophosome symbiont of *Riftia pachyptila*	M99451	Tube worm (*Riftia pachyptila*)	*Thiothrix nivea*	90.0 %	Distel et al., 1988
Mussel methanotrophic gill symbiont MAR2	U29164	Hydrothermal vent mussel	*Methylobacter luteus*	92.8 %	Distel et al. 1995
Endosymbiont of another bacterial endosymbiont	AF322016	Betaptoteobacterial endosymbiont [a]	*Brenneria quercina*	93.6 %	von Dohlen et al., 2001
Candidatus Devosia euplotis	AJ548825	Ciliate (*Euplotes magnicirratus*)	*Devosia riboflavina*	95.9 %	Vannini et al., 2004
Secondary endosymbiont	M27040	Aphid (*Acyrthosiphon pisum*)	*Serratia plymuthica*	97.8 %	Unterman et al., 1989
Candidatus Burkholderia calva	AY277697	*Psychotria calva* (angiosperm plant)	*Burkholderia* sp. NF100	97.9 %	Van Oevelen et al., 2004
Polynucleobacter sp. Bod2	XXX	Ciliate (*Euplotes harpa*)	*Polynucleobacter* sp. MWH-Creno-4B4	99.5 %	this study
Polynucleobacter necessarius ATCC 30859^T	XXX	Ciliate (*Euplotes aediculatus*)	*Polynucleobacter* sp. MWH-Gad-W7	99.5 %	this study

[a] of mealybug (*Planococcus citri*)
[b] pathogenic bacterium

Table 3. Detection of free-living and endosymbiotic PnecC strains in the pelagic of freshwater lakes. Probe Omi10-mod is specific for the endosymbionts of *Euplotes aediculatus*. Probe PnecC-16S-445 is specific for the entire subcluster C (PnecC) of the *Polynucleobacter* cluster. This subcluster includes endosymbionts of at least two *Euplotes* species, as well as many environmental sequences, and sequences from freshwater isolates. All bacteria that hybridized with probe PnecC-16S-445 were detected outside of protist cells.

Habitat	Cell numbers [10^4 ml^{-1}]	
	PnecC-16S-445	Omi10-mod
Lake Mondsee	0.4	n.d. [1]
Lake Wolfgangsee[2]	0.6	n.d.
Lake Irrsee	1.9	n.d.
Lake Krottensee[2]	6.6	n.d.
Lake Egelsee (M2)[2]	8.4	n.d.
Pond Kleine Lacke	67.9	n.d.

[1] n.d. - no detection
[2] samples from the benthic zone of these habitats were used for attempts to isolate *Euplotes* spp.

Table 4. Methods and conditions used in the eight cultivation experiments performed with endosymbionts from two ciliate species.

Cultivation method	Initial Medium	Acclimatization Medium [a]	Endosymbionts of	Dilutions X Number of inoculated wells
Filtration, acclimatization	IBM medium [b]	NSY medium	*E. aediculatus* STIR1	3 X 24
			E. harpa FSP1.4	3 X 24
Dilution, acclimatization	IBM medium [b]	NSY medium	*E. aediculatus* STIR1	3 X 24
			E. harpa FSP1.4	3 X 24
Dilution, acclimatization	ciliate homogenate	NSY medium	*E. aediculatus* STIR1	3 X 8
Dilution, no acclimatization	NSY medium [c]	none	*E. aediculatus* STIR1	3 X 24
			E. harpa FSP1.4	3 X 24
Dilution, no acclimatization	ciliate homogenate [c]	none	*E. aediculatus* STIR1	6 X 4

[a] stepwise increase of concentration
[b] enriched with a low concentration of NSY medium
[c] no change of medium in the course of the experiment

Figure Legends

Fig. 1. Geographic location of the sites from which the five endosymbiont bearing *Euplotes* spp. strains (*E. aediculatus*, squares; *E. harpa*, triangles), and the 40 free-living *Polynucleobacter* sp. (circles) strains affiliated with subcluster PnecC were isolated. If more than one isolate was obtained from the same region, the number of habitats (first number) and the number of strains (second number) obtained from the respective region are indicated. The five *Euplotes* spp. cultures harboring the endosymbiotic strains were each obtained from habitats located in different regions. The European area (framed in (A)) from which the majority of investigated strains were obtained is shown in (B) with a higher magnification.

Fig. 2. Neighbor-joining tree based on almost complete 16S rRNA sequences (*E. coli* position 39 to 1542) of endosymbiotic (highlighted in bold) and free-living strains affiliated with subcluster C (PnecC) of the *Polynucleobacter* cluster. The tree was calculated with a set of sequences including the shown 45 sequences affiliated with subcluster PnecC, as well as sequences of twelve PnecD strains, one PnecB strain and one *Ralstonia solanacearum* strain (outgroup). The geographic origin of the isolates is indicated in brackets (E, Europe; As, Asia; O, Oceania; SA, South America). Bootstrap (1,000 iterations) values of >60% are shown. The scale bar indicates 1% estimated sequence divergence.

Fig. 3. Neighbor-joining tree based on complete 16S-23S internal transcribed spacer (ITS) sequences (approx. 500 bp) of endosymbiotic (highlighted in bold) and free-living strains affiliated with subcluster PnecC of the *Polynucleobacter* cluster. The tree was calculated with 59 sequences of the same organisms as used for the calculation of the 16S rRNA tree (Fig. 2). All other details as described for Fig. 2.

Fig. 4. Base pair mismatches in helices of the 16S rRNA as suggested by a secondary structure model provided by the software package ARB (Ludwig et al. 2004). The sequences of the three *E. harpa* endosymbionts differ significantly ($P<0.05$, one way ANOVA) in the number of base pair mismatches from all other groups (asterisk). Only sequences obtained by direct sequencing of the 16S rRNA gene were considered in this analysis. In total, 62 sequences were analyzed. Error bars depict standard deviation. PnecC - subcluster C, PnecD - subcluster D of the *Polynucleobacter* cluster (Hahn 2003), E. aed. – *E. aediculatus*, E. harp. – *E. harpa*.

Fig. 5. (A) Genome size of *Polynucleobacter* strains as determined by pulsed field gel electrophoresis. Data for endosymbiotic strains (grey bars), free-living PnecC strains (black bars), and free-living PnecD strains (hatched bars) are presented. Strains QLW-P1DMWA-1, QLW-P1FAT50D-2, and QLW-P1DNSYA-2 were isolated from the same habitat, share identical 16S rRNA and ITS sequences, as well as RAPD fingerprints. These three strains may represent the same clone. (B) Comparison of the genome size of the investigated *Polynucleobacter* strains and 22 other genome sequenced strains affiliated to the family *Burkholderiaceae*. These strains include members of the genera *Burkholderia*, *Ralstonia* and *Cupriavidus*. Average genome sizes and standard deviations are shown for the two groups. Genome size data of strains affiliated to the other five genera of the family are currently not available.

Fig. 6. G+C content of the entire genomes (A) and of the 16S-23S ITS (B) of endosymbiotic and free-living *Polynucleobacter* strains, as well as of other members of the family *Burkholderiaceae*. The genomic G+C content of the *P. necessarius* type strain (endosymbiont of *E. aediculatus*) was determined by Heckmann & Schmidt (1987), and the genomic G+C

content of the free-living strain was determined by genome sequencing. The genomic G+C content of 28 other *Burkholderiaceae* strains affiliated to five different genera was teken from the literature, and is presented as average and standard deviation. The analysis of the G+C content of the ITS sequences was restricted to ITS loci including tRNA-Ala and tRNA-Ile genes.

Fig. 1.

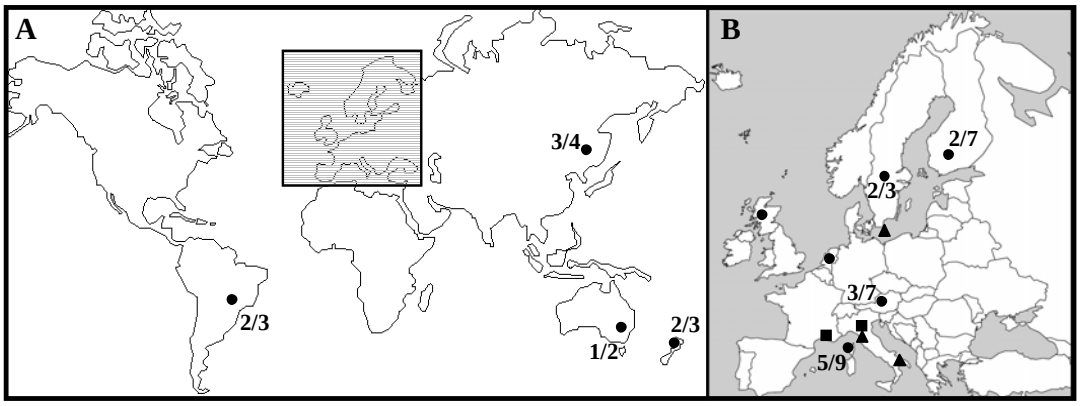


Fig. 2

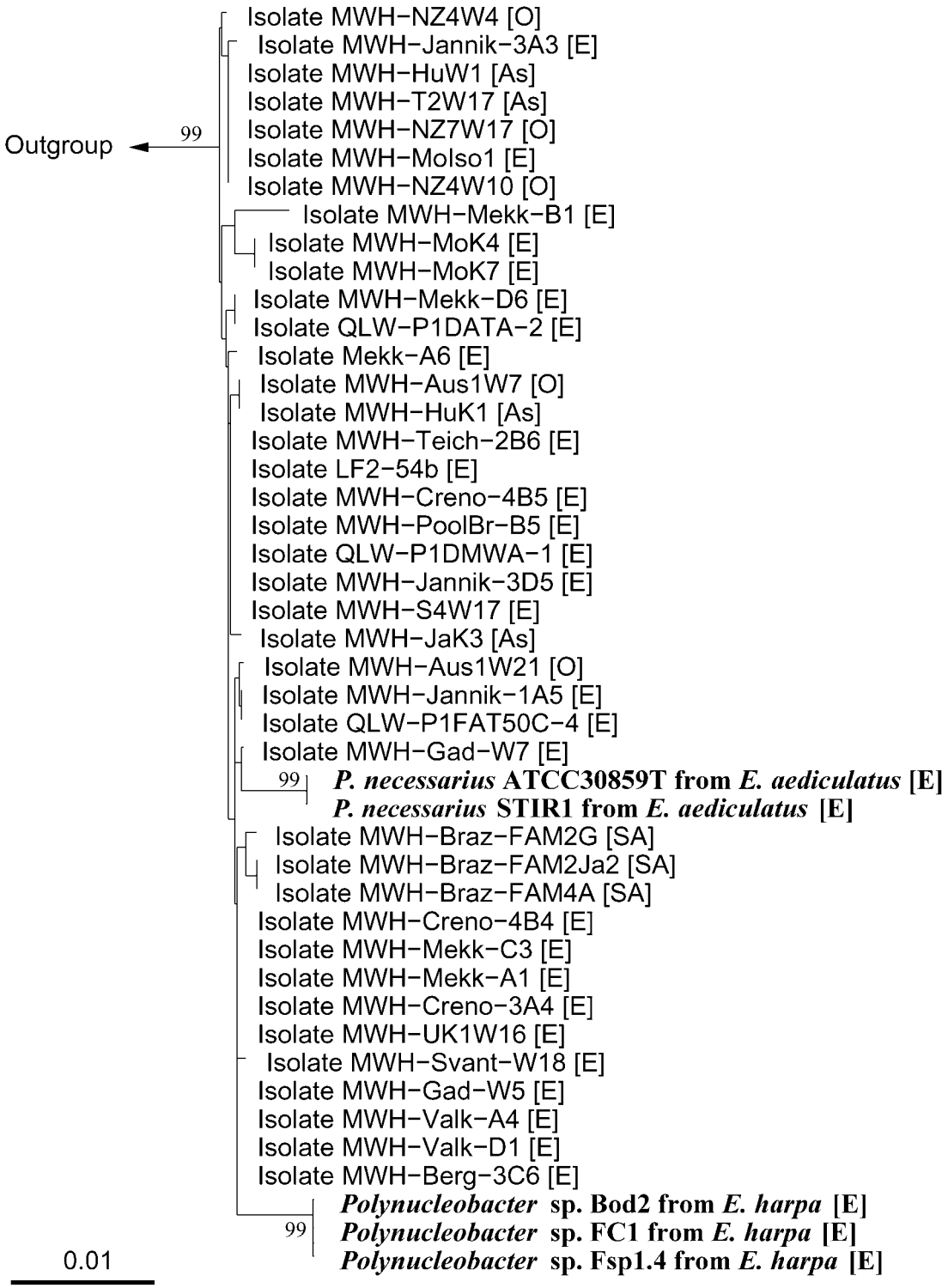

Outgroup
99
Isolate MWH-NZ4W4 [O]
Isolate MWH-Jannik-3A3 [E]
Isolate MWH-HuW1 [As]
Isolate MWH-T2W17 [As]
Isolate MWH-NZ7W17 [O]
Isolate MWH-Molso1 [E]
Isolate MWH-NZ4W10 [O]
Isolate MWH-Mekk-B1 [E]
Isolate MWH-MoK4 [E]
Isolate MWH-MoK7 [E]
Isolate MWH-Mekk-D6 [E]
Isolate QLW-P1DATA-2 [E]
Isolate Mekk-A6 [E]
Isolate MWH-Aus1W7 [O]
Isolate MWH-HuK1 [As]
Isolate MWH-Teich-2B6 [E]
Isolate LF2-54b [E]
Isolate MWH-Creno-4B5 [E]
Isolate MWH-PoolBr-B5 [E]
Isolate QLW-P1DMWA-1 [E]
Isolate MWH-Jannik-3D5 [E]
Isolate MWH-S4W17 [E]
Isolate MWH-JaK3 [As]
Isolate MWH-Aus1W21 [O]
Isolate MWH-Jannik-1A5 [E]
Isolate QLW-P1FAT50C-4 [E]
Isolate MWH-Gad-W7 [E]
99
P. necessarius ATCC30859T from E. aediculatus [E]
P. necessarius STIR1 from E. aediculatus [E]
Isolate MWH-Braz-FAM2G [SA]
Isolate MWH-Braz-FAM2Ja2 [SA]
Isolate MWH-Braz-FAM4A [SA]
Isolate MWH-Creno-4B4 [E]
Isolate MWH-Mekk-C3 [E]
Isolate MWH-Mekk-A1 [E]
Isolate MWH-Creno-3A4 [E]
Isolate MWH-UK1W16 [E]
Isolate MWH-Svant-W18 [E]
Isolate MWH-Gad-W5 [E]
Isolate MWH-Valk-A4 [E]
Isolate MWH-Valk-D1 [E]
Isolate MWH-Berg-3C6 [E]
Polynucleobacter sp. Bod2 from E. harpa [E]
99
Polynucleobacter sp. FC1 from E. harpa [E]
Polynucleobacter sp. Fsp1.4 from E. harpa [E]
0.01

Fig. 3

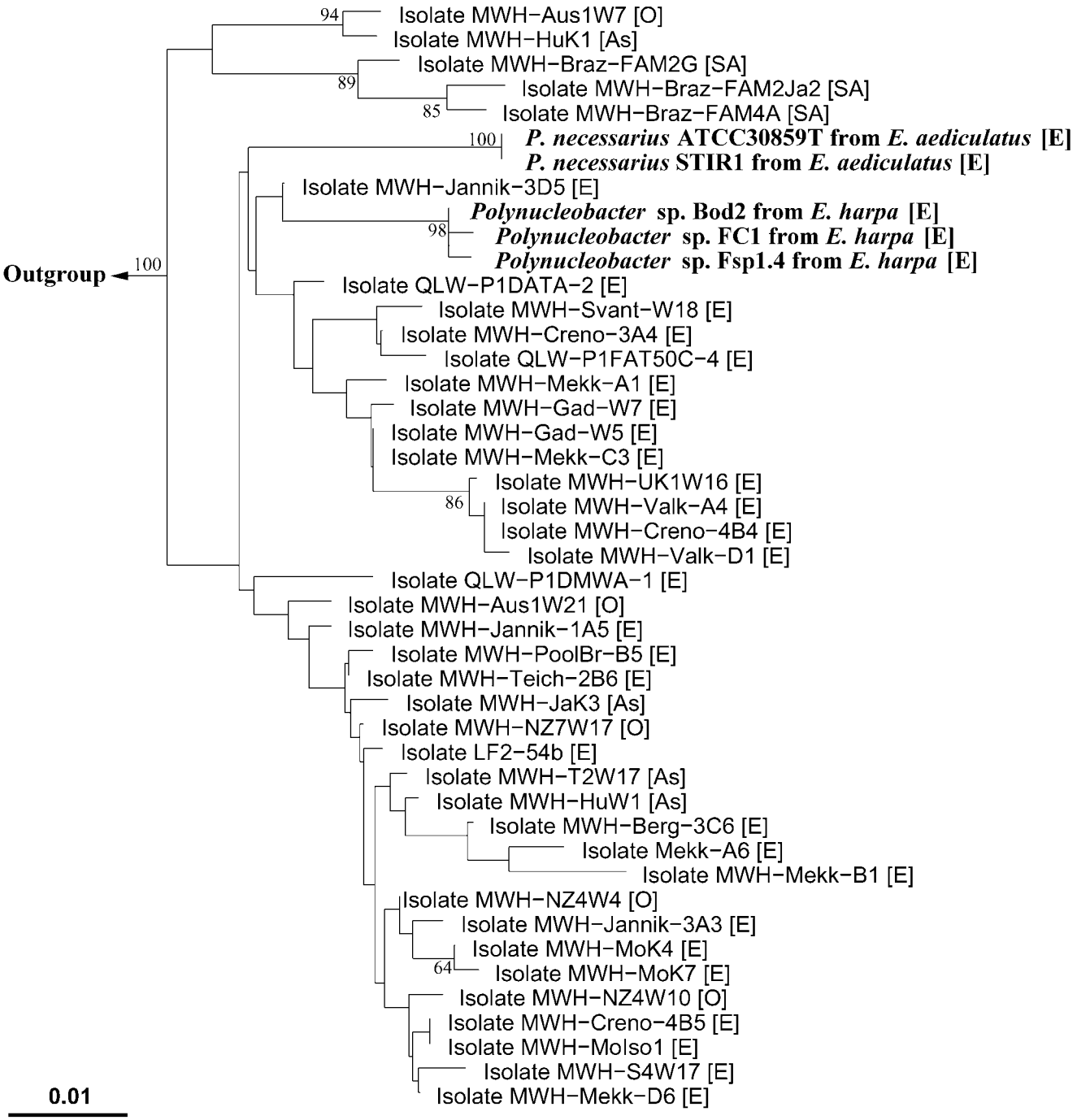

Isolate MWH-Aus1W7 [O]
Isolate MWH-HuK1 [As]
Isolate MWH-Braz-FAM2G [SA]
Isolate MWH-Braz-FAM2Ja2 [SA]
Isolate MWH-Braz-FAM4A [SA]
P. necessarius ATCC30859T from E. aediculatus [E]
P. necessarius STIR1 from E. aediculatus [E]
Isolate MWH-Jannik-3D5 [E]
Polynucleobacter sp. Bod2 from E. harpa [E]
Polynucleobacter sp. FC1 from E. harpa [E]
Polynucleobacter sp. Fsp1.4 from E. harpa [E]
Outgroup
Isolate QLW-P1DATA-2 [E]
Isolate MWH-Svant-W18 [E]
Isolate MWH-Creno-3A4 [E]
Isolate QLW-P1FAT50C-4 [E]
Isolate MWH-Mekk-A1 [E]
Isolate MWH-Gad-W7 [E]
Isolate MWH-Gad-W5 [E]
Isolate MWH-Mekk-C3 [E]
Isolate MWH-UK1W16 [E]
Isolate MWH-Valk-A4 [E]
Isolate MWH-Creno-4B4 [E]
Isolate MWH-Valk-D1 [E]
Isolate QLW-P1DMWA-1 [E]
Isolate MWH-Aus1W21 [O]
Isolate MWH-Jannik-1A5 [E]
Isolate MWH-PoolBr-B5 [E]
Isolate MWH-Teich-2B6 [E]
Isolate MWH-JaK3 [As]
Isolate MWH-NZ7W17 [O]
Isolate LF2-54b [E]
Isolate MWH-T2W17 [As]
Isolate MWH-HuW1 [As]
Isolate MWH-Berg-3C6 [E]
Isolate Mekk-A6 [E]
Isolate MWH-Mekk-B1 [E]
Isolate MWH-NZ4W4 [O]
Isolate MWH-Jannik-3A3 [E]
Isolate MWH-MoK4 [E]
Isolate MWH-MoK7 [E]
Isolate MWH-NZ4W10 [O]
Isolate MWH-Creno-4B5 [E]
Isolate MWH-Molso1 [E]
Isolate MWH-S4W17 [E]
Isolate MWH-Mekk-D6 [E]
94
89
85
100
98
100
86
64
0.01

Fig. 4

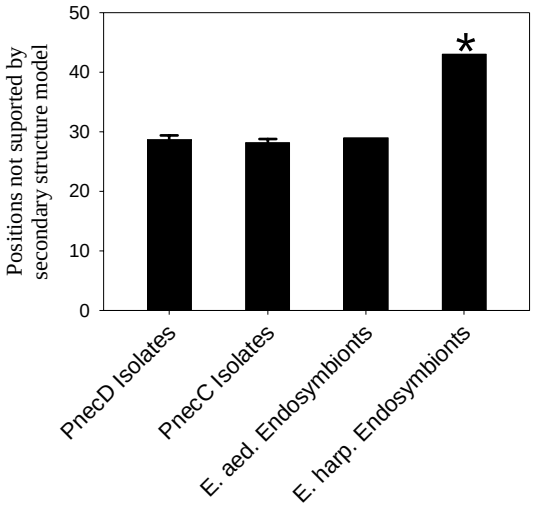

Positions not suported by secondary structure model
50
40
30
20
10
0
*
PnecD Isolates
PnecC Isolates
E. aed. Endosymbionts
E. harp. Endosymbionts

Fig. 5

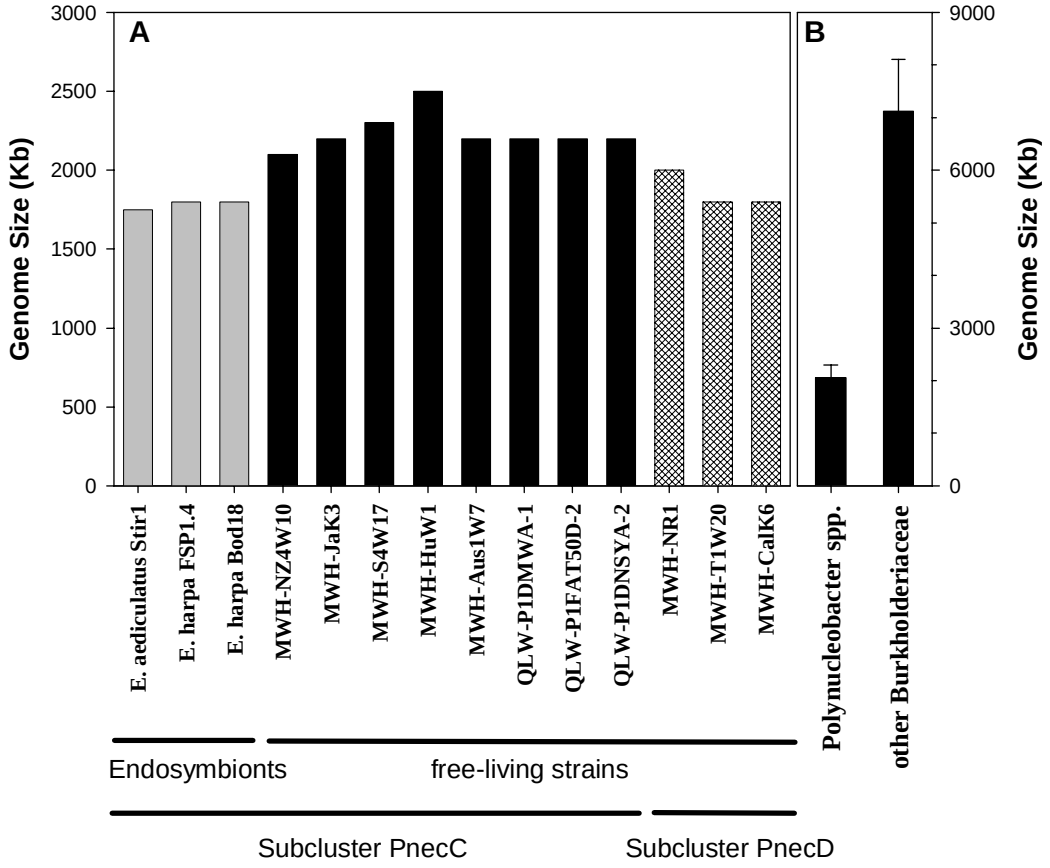
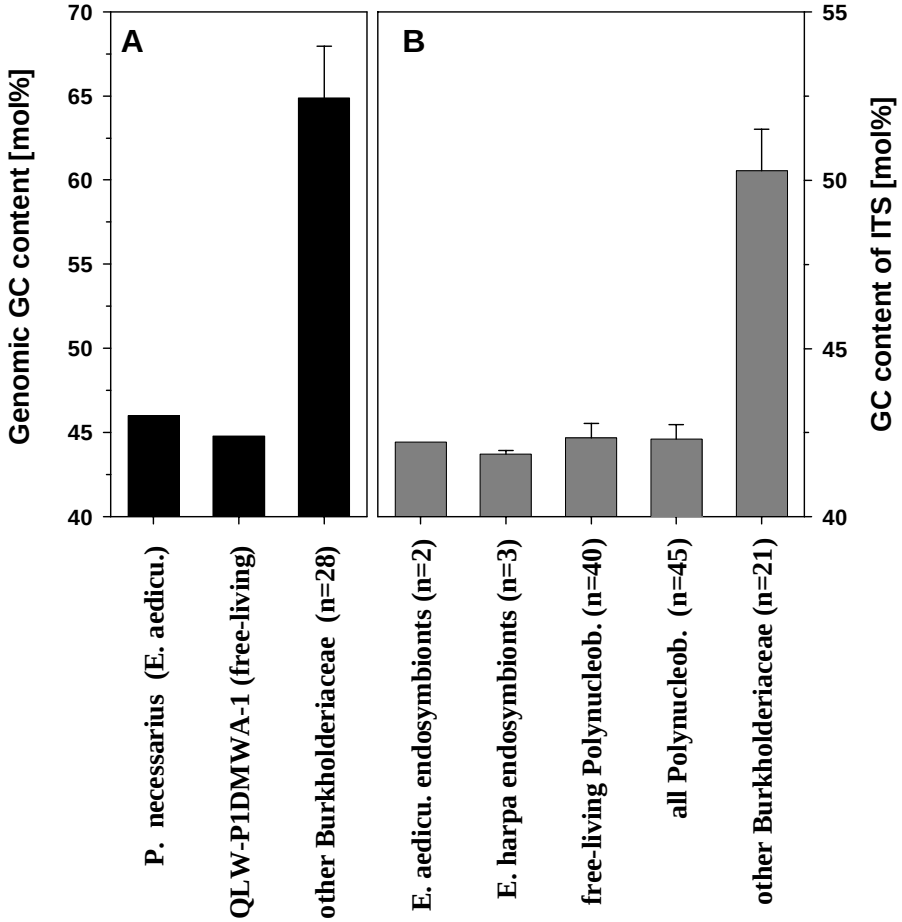

A
B
Genome Size (Kb)
3000
2500
2000
1500
1000
500
0
9000
6000
3000
0
E. aediculatus Stir1
E. harpa FSP1.4
E. harpa Bod18
MWH-NZ4W10
MWH-JaK3
MWH-S4W17
MWH-HuW1
MWH-Aus1W7
QLW-P1DMWA-1
QLW-P1FAT50D-2
QLW-P1DNSYA-2
MWH-NR1
MWH-T1W20
MWH-CalK6
Polynucleobacter spp.
other Burkholderiaceae
Endosymbionts
free-living strains
Subcluster PnecC
Subcluster PnecD

Fig. 6


A
B
Genomic GC content [mol%]
GC content of ITS [mol%]
70
65
60
55
50
45
40
55
50
45
40
P. necessarius (E. aedicu.)
QLW-P1DMWA-1 (free-living)
other Burkholderiaceae (n=28)
E. aedicu. endosymbionts (n=2)
E. harpa endosymbionts (n=3)
free-living Polynucleob. (n=40)
all Polynucleob. (n=45)
other Burkholderiaceae (n=21)