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ORIGINAL ARTICLE

The microbiome of North Sea copepods

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Abstract Copepods can be associated with different kinds and different numbers of bacteria. This was already shown in the past with culture-dependent microbial methods or microscopy and more recently by using molecular tools. In our present study, we investigated the bacterial community of four frequently occurring copepod species, *Acartia* sp., *Temora longicornis*, *Centropages* sp. and *Calanus helgolandicus* from Helgoland Roads (North Sea) over a period of 2 years using DGGE (denaturing gradient gel electrophoresis) and subsequent sequencing of 16S-rDNA fragments. To complement the PCR-DGGE analyses, clone libraries of copepod samples from June 2007 to 208 were generated. Based on the DGGE banding patterns of the two years survey, we found no significant differences between the communities of distinct copepod species, nor did we find any seasonality. Overall, we identified 67 phylotypes (>97 % similarity) falling into the bacterial phyla of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. The most abundant phylotypes were affiliated to the Alphaproteobacteria. In comparison with PCR-DGGE and clone libraries, phylotypes of the Gammaproteobacteria dominated the clone libraries, whereas Alphaproteobacteria were most abundant in the PCR-DGGE analyses.

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Introduction

Marine copepods may constitute up to 80 % of the mesozooplankton biomass (Verity and Smetacek 1996). They are key components of the food web as grazers of primary production and as food for higher trophic levels, such as fish (Cushing 1989; Møller and Nielsen 2001). Copepods contribute to the microbial loop (Azam et al. 1983) due to "sloppy feeding" (Møller and Nielsen 2001) and the release of nutrients and DOM from faecal pellets (Hasegawa et al. 2001; Møller et al. 2003; Steinberg et al. 2004). Similarly, their moults and carcasses can be populated and decomposed by bacteria (Tang et al. 2009b; Tang et al. 2006a, b).

Copepods are colonised by bacteria, especially around the oral region and the anus as well as the body appendages (Huq et al. 1983a), the intersegmental parts (Carman and Dobbs 1997) and the intestine. The nature of this association and also the origin of these bacteria are not well understood until now. To date, it is not known whether copepods exhibit a distinct bacterial community related to their lifestyle or metabolism or whether they simply represent polymeric chitinous surfaces in the marine environment. Bacteria located on the copepod exterior, in the gut, on faecal pellets and carcasses have been investigated with different methods in a number of previous studies (Sochard et al. 1979; Brandt et al. 2010; Carman 1994; Nagasawa et al. 1985; Nagasawa and Nemoto 1988; Delille and Razouls 1994; Hansen and Bech 1996; Carman and Dobbs 1997; Tang 2005; Møller et al. 2007; Tang et al. 2009b; Harding 1973; Nagasawa 1992; Kirchner 1995). However, these studies were only snap shots investigating

geographically different marine sites and several copepod genera by applying a vast variety of methods.

Historically, classical microbiological cultivation methods were used to obtain an insight into the bacterial community of some copepod genera (e.g. Sochard et al. 1979; DeLong et al. 1993; Heidelberg et al. 2002b). Molecular techniques that do not require cultivation recently opened new perspectives (Heidelberg et al. 2002b) in the analysis of the copepod-associated bacterial community (Møller et al. 2007). The authors identified bacteria predominantly affiliated with the Alphaproteobacteria. Since the majority of former studies focussed on the identification of copepod-associated pathogens, e.g., *Vibrio cholerae* (e.g. Huq et al. 1983; Tamplin et al. 1990; Huq et al. 1984; Heidelberg et al. 2002a; Kaneko and Colwell 1975; Belas and Colwell 1982; Nalin et al. 1979), to date not much is known about the total bacterial community associated with copepods or seasonal dynamics of the associated bacteria.

To close this gap in knowledge, the present study aims to describe the bacterial communities of four frequently occurring calanoid copepod species of the North Sea (*Acartia* sp., *Temora longicornis*, *Centropages* sp., *Calanus helgolandicus*) during two seasonal cycles using DGGE and subsequent 16S-rDNA sequencing.

Materials and methods

Sample collection and preparation

Zooplankton samples from Helgoland Roads in the North Sea (54°11.3′N and 7°54.0′E) were collected between February 2007 and March 2009 using a 150 µm and a 500 µm net aboard the research vessels "Aade" and "Diker". The sampling intervals were weekly (first 6 months), bi-weekly (second 6 months) and monthly (second year), respectively. Specimens from *Acartia* sp. (ACA), *Temora longicornis* (TEM), *Centropages* sp. (CEN) and *Calanus helgolandicus* (CAL) were sampled during the two years. Using a stereo microscope, the animals were sorted by genus with sterile tweezers and washed twice with sterile sea water. For the genera *Acartia* and *Centropages,* the exact species affiliation is hard to determine. Hence, we decided to denominate *Acartia* and *Centropages* specimens by *Acartia* sp. and *Centropages* sp., respectively. Until further analysis, individual copepods were frozen in sterile reaction tubes at−20 °C.

DNA extraction

DNA extraction was carried out using a pellet pestle (Kontes, Vineland, NJ, USA). Bundles of three (CAL) or five (all other copepod genera) copepod individuals were ground for 30 s, respectively. This was followed by a phenol–chloroform-DNA extraction with SDS and lysozyme as described elsewhere (Brandt et al. 2010).

PCR

PCR amplification of 16S rRNA gene fragments for DGGE was performed using the bacteria-specific primers 341 with GC-clamp (P3) (Muyzer et al. 1993) with a 40-bp GC-rich sequence at the 5′ end and 907r (Muyzer et al. 1995).

PCR mixtures with a volume of 50 µl contained 5 µl of 10× Taq buffer (5 Prime, Hamburg, Germany), 8 µl of Master Enhancer (5 Prime) for initial PCRs and no enhancer for re-amplification after DGGE, 200 µM dNTPs (Promega, Mannheim, Germany), 0.2 µM of each primer, 2 U of Taq DNA polymerase (5 Prime) and 2 µl of DNA prior and after DGGE. "Touchdown"-PCR was performed as described elsewhere (Sapp et al. 2007). PCRs were conducted in a Mastercycler (Eppendorf, Hamburg, Germany), and PCR products were separated on 1.2 % (w/v) agarose gels (50 min at 100 V in 0.5× TBE).

For the cloning approach, the primers 63f and 1387r (Marchesi et al. 1998) were used for PCR (composition of PCR mixtures, see above). The temperature profile was as follows: 5 min initial denaturing at 94 °C, 30 cycles with denaturing at 94 °C for one min, annealing at 55 °C for one min and elongation at 68 °C for two min was followed by a final elongation step at 68 °C for 6 min.

Separated PCR products were visualised by ethidium bromide (0.5 mg l^{-1}), and images were captured with a ChemiDoc XRS System (BioRad, München, Germany). The thickness and intensity of each band visualised were used to gauge the relative volume of the corresponding product used for DGGE (see below).

DGGE

All 16S rRNA gene amplicons were resolved on 6 % (w/v) polyacrylamide gels in 0.5× TAE buffer (20 mM TrisHCl, 10 mM acetic acid, 0.5 mM EDTA) with denaturing gradient of 15–55 % urea/formamide (100 % denaturant contains 7 M urea and 40 % formamide). Electrophoresis was performed at 60 °C and 150 V for 10 h (Sigler et al. 2004) using a DCode mutation detection system (BioRad). DGGE gels were stained with SYBRGold (Invitrogen, Karlsruhe, Germany). Imaging was performed with a ChemiDoc XRS System (BioRad). Prominent DGGE bands were excised, eluted in 50 µl PCR-water (Eppendorf) by gently shaking at 37 °C for 60 min, re-amplified and confirmed by an additional DGGE. As a marker for comparative analyses of all DGGE gels, the combined PCR-amplicons (GC-341f/907r) of four bacteria (*Polaribacter*

filamentus DSM 13964, *Sulfitobacter mediterraneus* DSM 12244, *Arthrobacter agilis* DSM 20550, *Leifsonia aquatica* DSM 20146) were used.

Multivariate analyses

Analyses of DGGE gel images were carried out with Bionumerics 5.0 software package (Applied Maths NV, Sint-Martens-Latem, Belgium). Normalisation of DGGE gels was performed using a marker consisting of combined PCR-amplicons (GC-341f/907r) of four bacteria with different GC-contents (see above). For sample comparison, band-matching analysis was performed, and bands were assigned to classes common within all profiles leading to a band-matching table.

PRIMER 5 software suite (PRIMER-E Ltd., Plymouth, UK) was used to calculate Jaccard similarities between samples, for nonmetric multidimensional scaling (nMDS) and analysis of similarities (ANOSIM). ANOSIM was applied for the grouping factors "genus" and "season".

Cloning

The 1,324 bp 16S rDNA-PCR fragment of selected copepod samples of *Acartia* sp. (ACA), *Temora longicornis* (TEM), *Centropages* sp. (CEN) and *Calanus helgolandicus* (CAL) of two dates, June 2007 (05.06.2007) and 2008 (03.06.2008), were cloned into the cloning vector PCR® 4-TOPO® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Via single-base sequencing, the clones were differentiated into different clone types (Schmidt et al. 1991). Single-base sequencing was conducted with DNA Sequenzer LONG READER IR4200 (LI-COR, Lincoln, NE, USA) and SequiTherm EXCEL™ II DNA Sequencing Kit LC (Epicentre, Madison, WI, USA) according to the manufacturer's protocol. Banding patterns with a similarity of more than 99 % were combined to one clone type.

DNA Sequencing and phylogenetic analysis

For sequencing, eluted DGGE bands were re-amplified using the primers 341f (without GC-clamp), and 907r. PCR products were checked on 1.2 % (w/v) agarose gels prior to sequencing. PCR products with the correct size ($\sim$566 bp) were excised from the agarose gels and used for sequencing.

The PCR products of different clone types were re-amplified with the primer pair 63f and 1387r and purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

DNA sequencing of PCR products was performed by Qiagen GmbH using an ABI PRISM 3700 DNA Analyser (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same as used for re-amplification. Sequence data were checked for the presence of PCR-amplified chimeric sequences by the CHECK_CHIMERA programme (Cole et al. 2003). Nearest relatives for all sequences were searched using BLAST (http://www.ncbi.nlm.nih.gov; Altschul et al. 1997). The ARB software package (http://www.arb-home.de) was used for phylogenetic analysis (Ludwig et al. 2004). After addition of sequences to the ARB 16S rRNA sequences database (SSU_ref database, release May 2007), alignment was carried out with the Fast Aligner integrated in the programme and refined by comparison of closest relatives retrieved by BLAST. Sequences with more than 1,300 nucleotides were used to calculate phylogenetic trees. The ARB "parsimony interactive" tool was used to add partial sequences to respective trees. Phylogenetic relationships were deduced by the neighbour-joining method including the correction algorithm of Felsenstein (1993).

Definition of phylotypes

Similarity values were calculated for all retrieved DNA sequences (excised DGGE bands and clone types) using the ARB tool similarity matrix (Ludwig et al. 2004). These values were used to assign DNA sequences to individual phylotypes based on 97 % sequence similarity.

Nucleotide sequence accession numbers

The sequences obtained in this study are available from GenBank under the accession numbers JX435603-JX435770 (partial SSU rRNA gene).

Results

Comparison of DGGE banding patterns

Overall, more than 2,000 copepod individuals were sampled over the two years. For DGGE analyses, we pooled three (CAL) to five copepod individuals (all others) from the same genus of one sampling day and obtained DGGE patterns from 105 different samples of the frequently occurring copepods. For all investigated copepod genera, between two to sixteen DGGE bands were observed.

For multivariate analyses of ACA, TEM, CEN and CAL samples, a total of 37 band classes were assigned. A few bands were omitted from multivariate analysis because they appeared outside of the marker band positions, but were included in the phylogenetic analysis.

Analysis of similarities (ANOSIM) showed no significant differences among the DGGE patterns of different copepod genera (global $R = 0.021$; $p = 20.6$ %) or

seasons (global $R = 0.017$; $p = 21.1$ %) nor any seasonal differences for the communities of the different genera (ACA: global $R = 0.009$; $p = 42$ %; TEM: global $R = 0.007$; $p = 41.3$ %; CEN: global $R = -0.021$; $p = 56.6$ %; CAL: global $R = 0.127$; $p = 4.7$ %). In Fig. 1a and b, two-dimensional nMDS plots of DGGE fingerprints according to the grouping factors "genus" and "season" are shown. The high stress value of 0.27 indicates that the ordination is almost arbitrary.

Analysis of 16S rDNA sequences from four copepod species

Overall, 238 16S rDNA fragments (201 DGGE bands and 37 clone types, retrieved from 432 clones which were checked for identity by single-base pair sequence analysis) were successfully sequenced. All sequences with similarity of >97 % were combined to distinct phylotypes (Table 1). By this procedure, we identified a total of 71 bacterial phylotypes, 40 different bacterial phylotypes for ACA, TEM, CEN and CAL by DGGE approach and 37 phylotypes from analysis of all clone types.

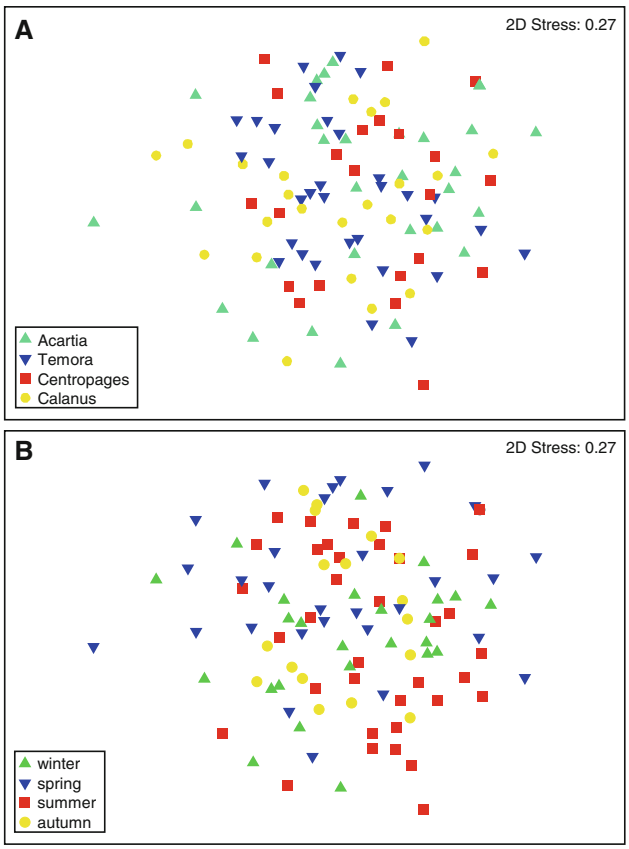


Fig. 1 **a** nMDS plot based on Bray–Curtis similarities of DGGE fingerprints of bacterial communities of four different copepod genera (*Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*). **b** nMDS plot based on Bray–Curtis similarities of DGGE fingerprints of bacterial communities of four different copepod genera (*Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*) during different seasons

Analysis of excised 16S rDNA DGGE bands

From DGGE band-sequencing approach, we identified a total of 40 different bacterial phylotypes for ACA, TEM, CEN and CAL (Table 1). Besides the bacterial sequences, one sequence matching with 18S rDNA of *Calanus* sp. (from CAL) was found.

Overall, the obtained bacterial phylotypes fell into four different bacterial phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Table 1). The phylum Firmicutes was absent only from CEN. In all cases, Proteobacteria was the dominating phylum.

We identified nine different bacterial classes: Actinobacteria, Bacilli and Clostridia (Fig. 2), Deltaproteobacteria, and Flavobacteria (Fig. 3), Alphaproteobacteria (Fig. 4, 5a, b), Betaproteobacteria, Sphingobacteria and Gammaproteobacteria (Fig. 6). In contrast to the other copepod genera, only three bacterial classes were detected for CEN (Alphaproteobacteria, Actinobacteria and Flavobacteria). In all cases, Alphaproteobacteria was the most abundant class. Altogether, we detected twelve different bacterial orders with the Rhodobacterales being the dominating order. Sphingobacteriales were only found associated with TEM and CAL. Rhizobiales were found exclusively associated with ACA and TEM, whereas the Sphingomonadales were discovered only associated with ACA. Burgholderiales were not detected in CAL samples, whereas only in these samples were Vibrionales observed. CEN samples only contained Actinomycetales, Flavobacteriales and Rhodobacterales. The orders could be separated into 18 different bacterial families. ACA harboured the highest number of different bacterial families (twelve), whereas ten families were associated with TEM. Ten different bacterial families were also associated with CAL and only four with CEN. The Rhodobacteraceae dominated in all four different copepod genera with 15 different phylotypes.

Overall, we identified 26 different bacterial genera associated with the four copepod species, belonging to Saprospiraceae, Rhodobacteriaceae and Lachnospiraceae, while four remained unclassified.

Analysis of clone libraries

For cloning, we used bundles of five copepod individuals of three copepod genera from two sampling dates in June 2007 and 2008 and collected 268 clones for *Acartia* sp., *T. longicornis* and *Centropages* sp.in 2007, and 164 clones in 2008. Altogether, 37 phylotypes could be identified (Table 1).

Table 1 Bacterial phylotypes associated with four different copepod genera (*Acartia* sp., *Temora longocornis*, *Centropages* sp. and *Calanus helgolandicus*)

PT	DGGE	Cloning	TEM	ACA	CAL	CEN	Phylum	Class	Order	Family	Genus
1	•					•	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	***Arthrobacter***
2		•		•							***Kocuria***
3	•	•	•	•						Intrasporangiaceae	***Ornithinimicrobium***
4	•			•	•					Microbacteriaceae	***Microbacterium***
5	•		•		•	•			Propionibacteriales	Propionibacteriaceae	***Propionibacterium***
6		•	•						Acidimicrobiales	Acidimicrobiaceae	uncultured
7		•	•								uncultured
8		•	•							Laminaceae	uncultured
9	•		•	•	•	•	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	***Polaribacter***
10		•	•								***Polaribacter***
11		•	•								***Gramella***
12	•	•	•								***Marixanthomonas***
13	•				•					Cryomorphaceae	***Fluviicola***
14	•		•					Sphingobacteria	Sphingobacteriales	Saprospiraceae	uncultured
15	•				•						***Lewinella***
16	•		•								***Lewinella***
17		•	•					Bacteroidia	Bacteroidales	Prevotellaceae	***Prevotella***
18	•				•		Firmicutes	Bacilli	Bacillales	Family XII Incertae Sedis	***Exiguobacterium***
19	•		•							Bacillaceae	***Bacillus***
20	•	•	•	•						Staphylococcaceae	***Staphylococcus***
21	•				•			Clostridia	Clostridiales	Lachnospiraceae	***unknown***
22	•	•		•			Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	uncultured
23	•	•	•								***Marinosulfonomonas***
24	•	•	•	•	•	•					***uncultured Sulfitobacter***
25	•	•	•	•	•	•					***Sulfitobacter***
26	•		•	•							uncultured
27	•		•								***Roseovarius***
28	•		•	•	•						***Roseovarius***
29	•					•					***Roseovarius***
30	•		•								Roseobacter clade NAC 11_7
31	•		•								***Ruegeria***
32	•			•							***Ruegeria***
33	•		•		•						***Oceanicola***
34	•				•						***Octadecabacter***
35	•	•	•	•		•					***Loktanella***
36		•	•								***Loktanella***
37		•	•								***Jannaschia***
38		•	•								***Ahrensia***
39	•			•							***Paracoccus***
40		•		•		•			Rhizobiales	Phyllobacteriaceae	***Hoeflea***
41		•		•					Sphingomonadales	Sphingomonadaceae	***Sphingopyxis***
42	•			•							***Sphingopyxis***
43		•		•							***Sphingomonas***
44	•			•							***Sphingomonas***
45	•			•						Erythrobacteraceae	***Erythrobacter***
46	•		•	•					Rhizobiales	Methylobacteriaceae	***Methylobacterium***
47		•	•						Caulobacterales	Caulobacteraceae	***Caulobacter***
48	•				•				Ricketsiales	Rickettsiaceae	***Rickettsia***
49		•	•					Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	***Bdellovibrio***
50	•			•							***OM27 clade***
51		•	•	•		•		Gammaproteobacteria	Vibrionales	Vibrionaceae	***Photobacterium***
52		•	•								***Aliivibrio***
53		•		•		•					***Vibrio***
54	•				•						***Vibrio***
55		•		•							***Vibrio***
56		•	•								***Vibrio***
57		•	•						Enterobacteriales	Enterobacteriaceae	***Hafnia***
58		•	•	•					Alteromonadales	Pseudoalteromonadaceae	***Pseudoalteromonas***
59		•		•						Alteromonadaceae	***Microbulbifer***
60	•		•	•					Thiotrichales	Thiotrichaceae	***Thiotrix***
61		•	•						Legionellales	Legionellaceae	***Legionella***
62	•	•	•	•		•			Pseudomonadales	Moraxellaceae	***Psychrobacter***
63	•	•	•	•							***Psychrobacter***
64		•				•					***Psychrobacter***
65		•	•						Oceanospirillales	Halomonadaceae	***Cobetia***
66	•	•	•	•		•		Betaproteobacteria	Burkholderiales	Comamonadaceae	***Delftia***
Σ	40	37	41	30	14	13	4	10	22	31	66
unique PTs	29	26									
shared PTs	10										

Fig. 2 Phylogenetic tree of Actinobacteria and Firmicutes. Sequences given in *bold* obtained from cloning approach and from PCR-DGGE analysis. Bootstrap values under 50 % are not displayed

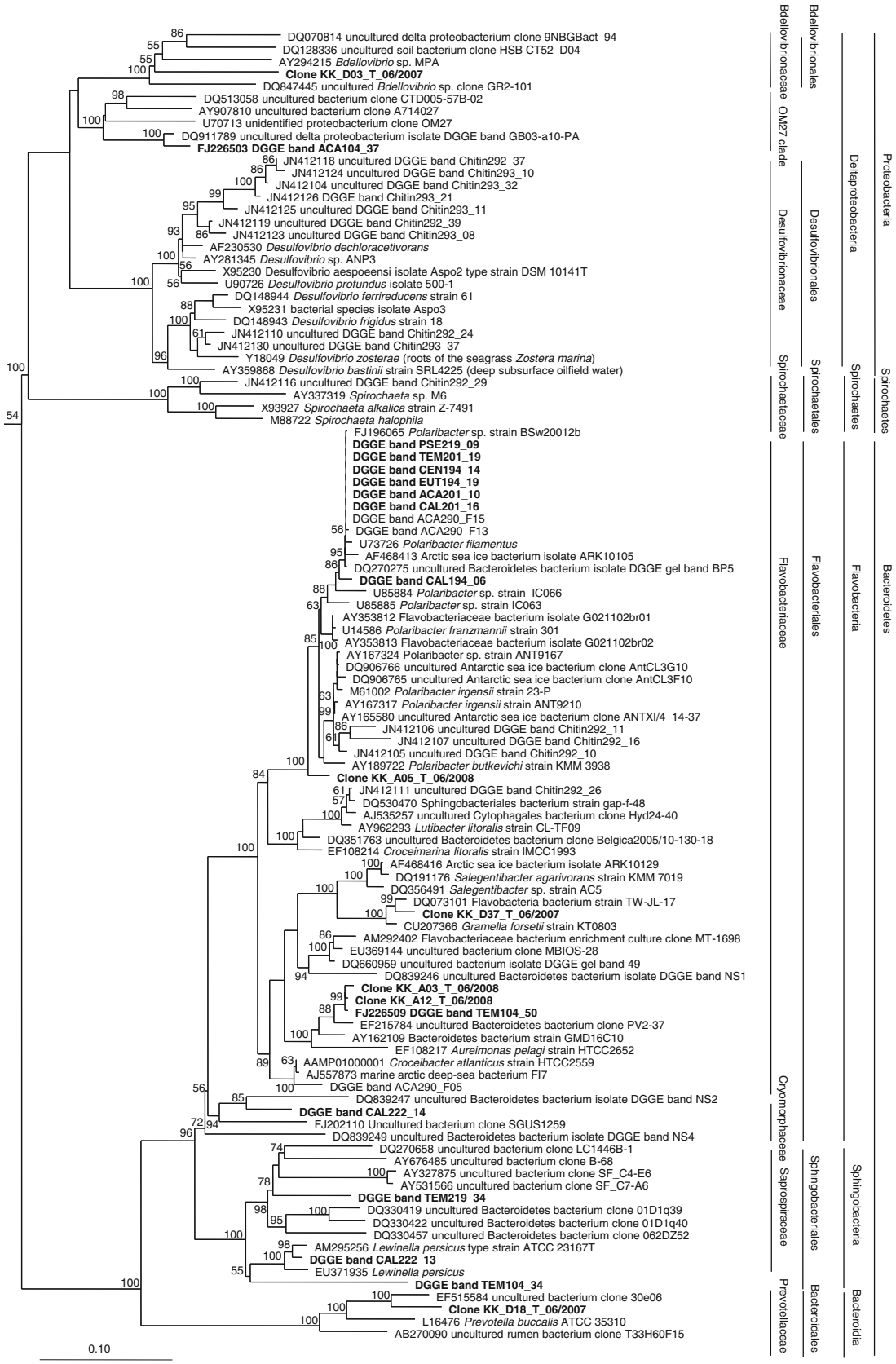


Fig. 3 Phylogenetic tree of Deltaproteobacteria, Bacteroidetes and Spirochaetes. In *bold* sequences obtained from cloning approach and from PCR-DGGE analysis. Bootstrap values under 50 % are not displayed

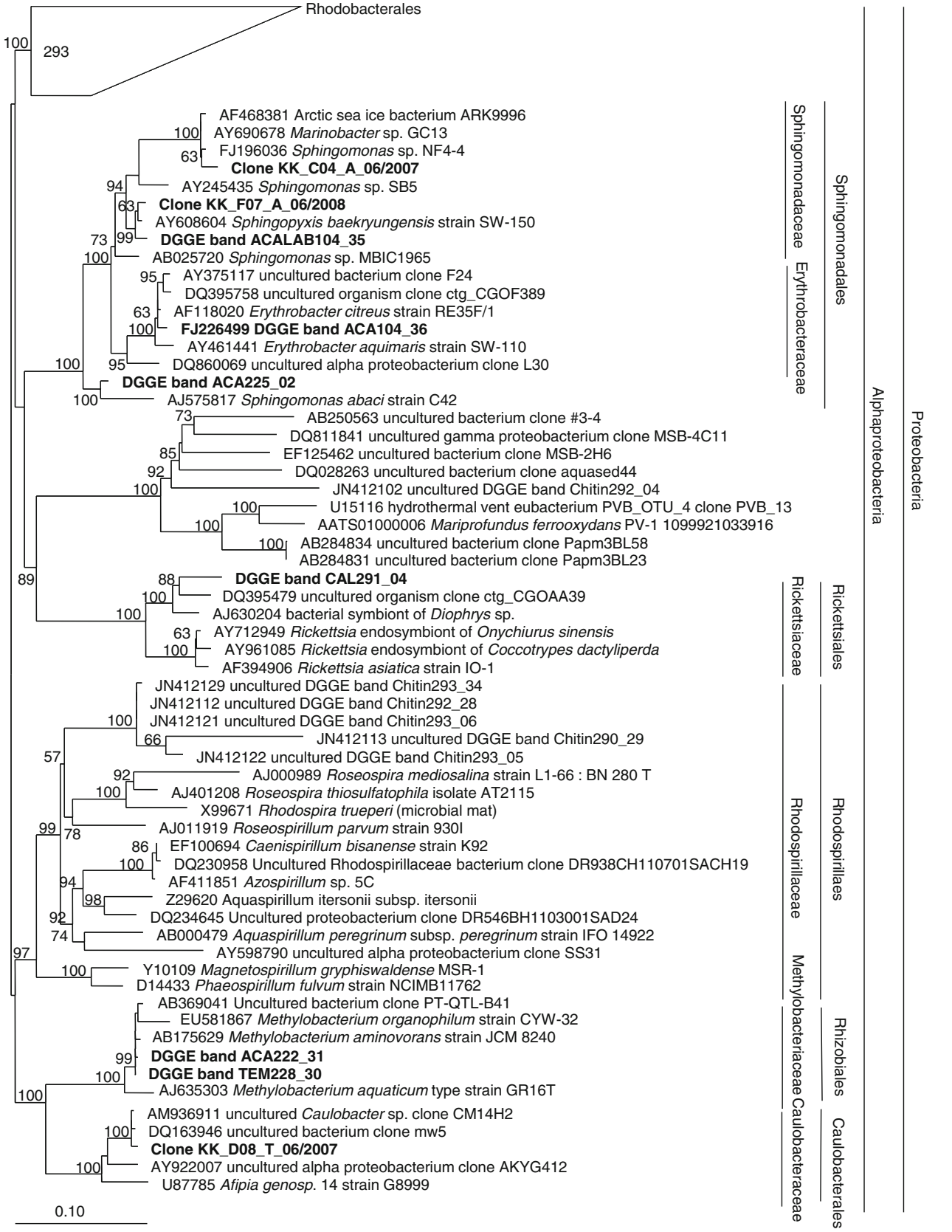


Fig. 4 Phylogenetic tree of Alphaproteobacteria without Rhodobacterales. In *bold* sequences obtained from cloning approach and from PCR-DGGE analysis. Bootstrap values under 50 % are not displayed

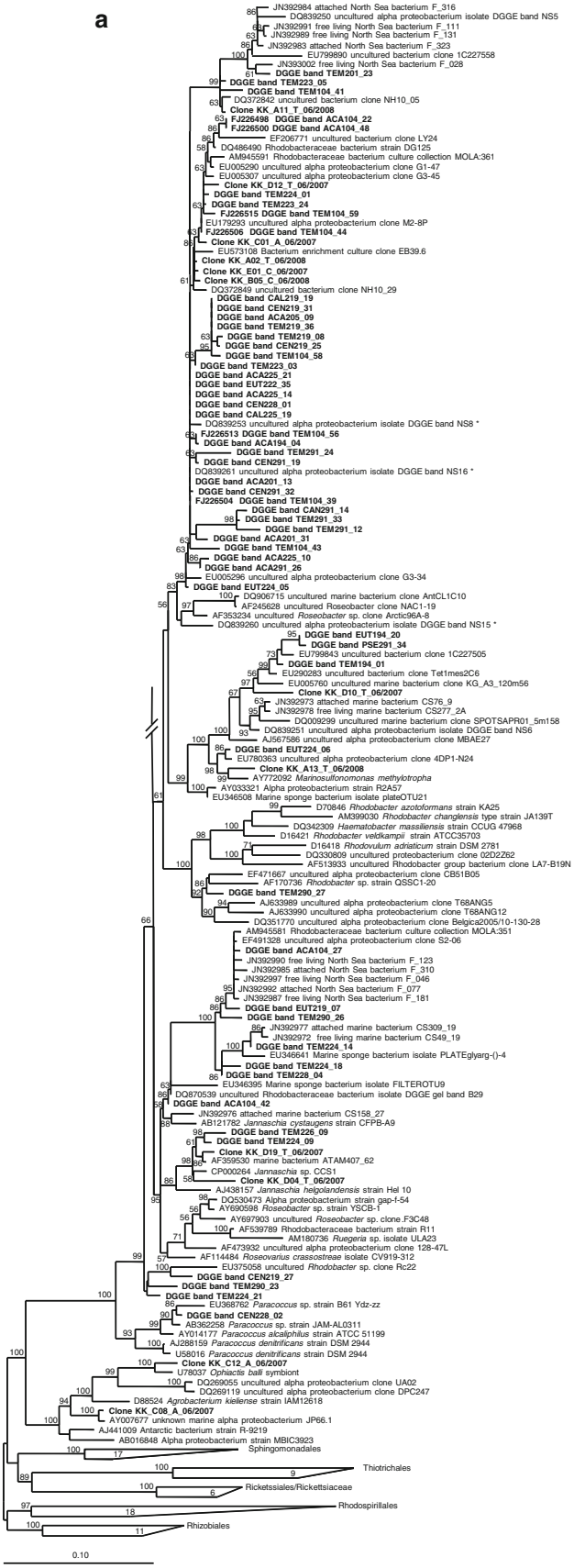
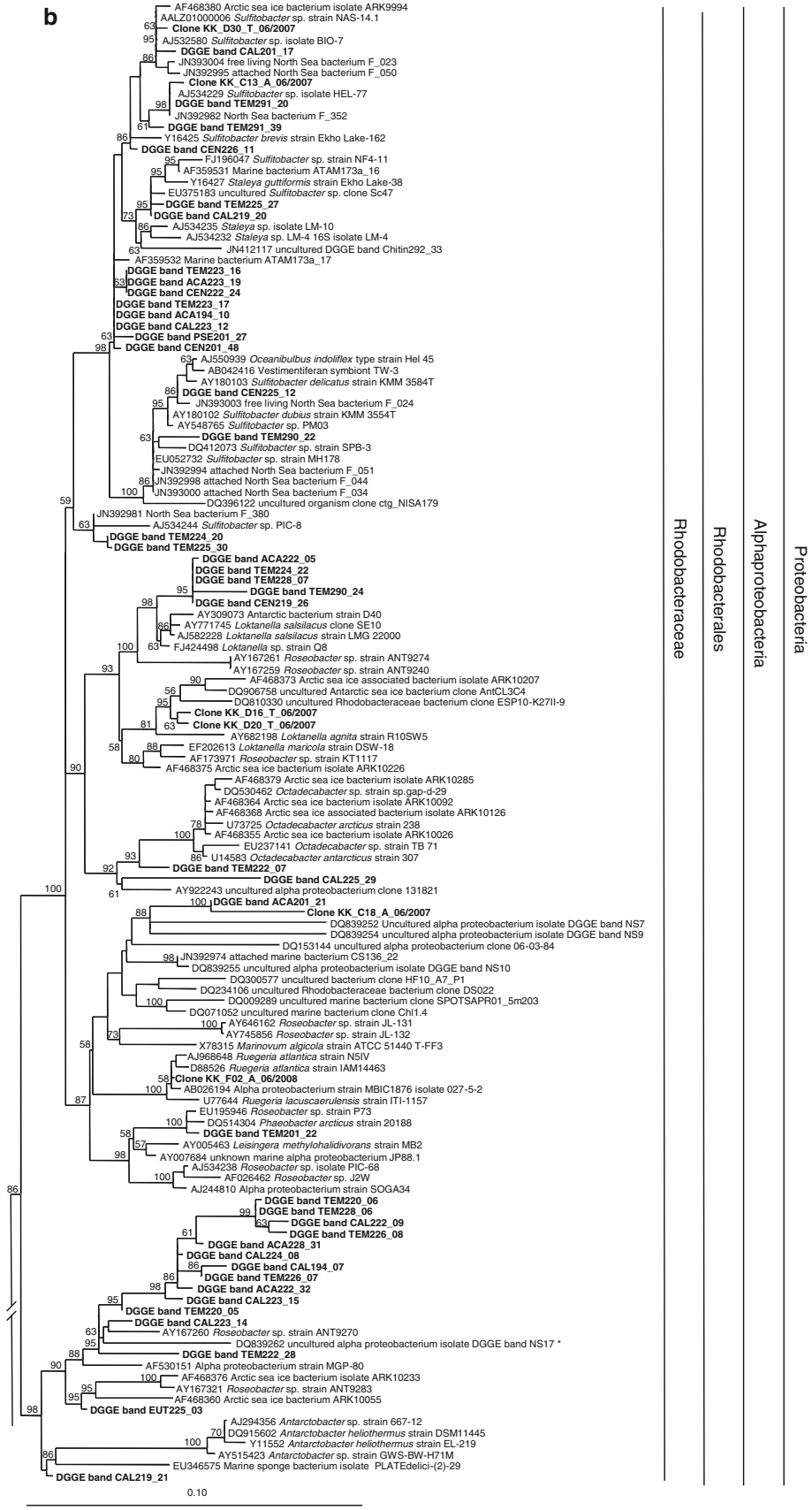


Fig. 5 **a**: Phylogenetic tree of Rhodobacterales. Sequences given in *bold* obtained from cloning approach and from PCR-DGGE analysis. Bootstrap values under 50 % are not displayed. **b** Phylogenetic tree of Rhodobacterales (continued). Twenty-five sequences given in *bold* obtained from cloning approach and from PCR-DGGE analysis. Bootstrap values under 50 % are not displayed

Fig. 5 continued



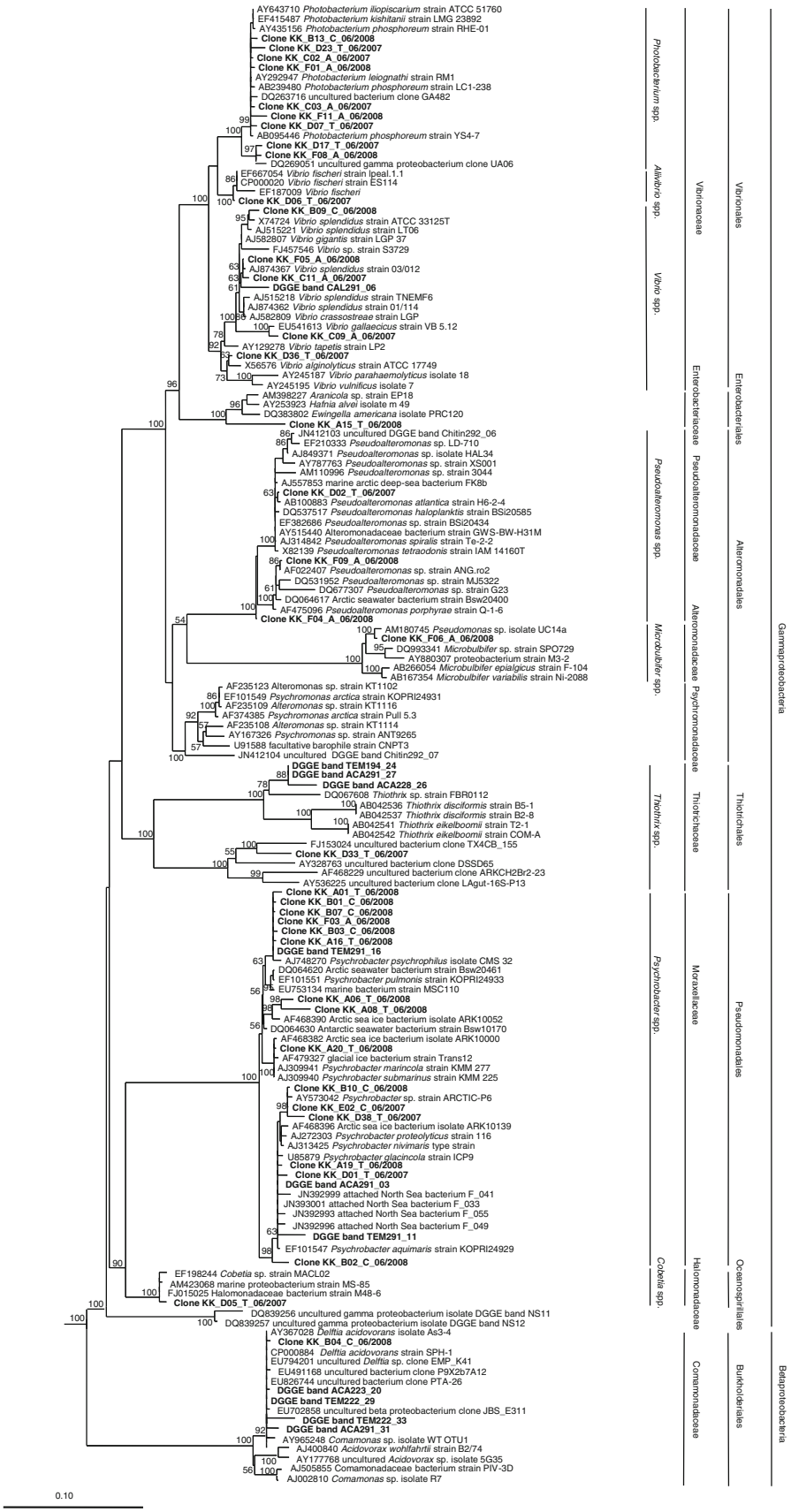


Fig. 6 Phylogenetic tree of Gamma-and Betaproteobacteria. In *bold* sequences obtained from cloning approach and from PCR-DGGE analysis. Bootstrap values under 50 % are not displayed

As compared to DGGE, we found four different bacterial phyla in the clone libraries: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Proteobacteria were the dominating phylum. The low bacterial diversity of *Centropages* sp.-samples is reflected also in the respective clone library. On the class level, we identified eight bacterial classes. Sphingobacteria and Clostridia were not discovered, but Bacteroidia occurred, which were not discovered by DGGE. In contrast to DGGE-sequencing results, more Gammaproteobacteria were detected.

Within the 8 bacterial classes, 17 orders of bacteria were identified. In addition to those detected by DGGE, Acidimicrobiales within the Actinobacteria and Bacteroidales as member of the Bacteroidia were discovered. Furthermore, we detected Caulobacterales affiliated to the Alphaproteobacteria, Bdellovibrionales which belong to the Deltaproteobacteria and Alteromonadales, Enterobacteriales, Legionellales as well as Oceanospirillales, which represented the Gammaproteobacteria (Fig. 6; Table 1). Within the clone libraries, the Pseudomonadales and Vibrionales dominated. Nineteen different bacterial families were identified. We found Lamiaceae as members of the Acidimicrobiales (Fig. 2; Table 1), Prevotellaceae which belong to the Bacteroidales (Fig. 3; Table 1) and Caulobacteraceae which fell into the Caulobacterales (Fig 4; Table 1). Pseudoalteromonadaceae of the Alteromonadales, and Enterobacteriaceae as representatives of Enterobacterales (Fig. 6), were found in the clone libraries. Halomonadaceae which belong to the Oceanospirillales (Fig. 6) as well as Phyllobacteriaceae which fell into the Rhizobiales (Fig. 4) were identified. We also detected the Bdellovibrionales-members Bdellovibrionaceae (Fig. 3) and Alteromonadaceae which fell into the Alteromonadales (Fig. 6), in addition to the families detected by DGGE. Dominating families as determined by cloning were Moraxellaceae and Vibrionaceae.

On the genus level, 23 different bacterial genera could be distinguished. Within the Micrococcaceae, the genus, *Kocuria*, was identified as well as *Gramella, Polaribacter* and *Marixanthomonas* as members of the Flavobacteriaceae. We found *Prevotella* of the family Prevotellaceae and *Hoeflea* of the family Phyllobacteriaceae and Staphylococcus of the family Staphylococcaceae. *Loktanella, Jannaschia and Ahrensia* as members of the Rhodobacteraceae (Fig. 5a, b) were identified by clone library analysis. *Bdellovibrio*, *Microbulbifer*, *Pseudoalteromonas, Legionella, Cobetia* as members of the families Bdellvibrionaceae, Alteromonadaceae, Pseudoalteromonadaceae, Legionellaceae and Halomonadaceae, respectively, were identified as well as the genera *Aliivibrio* and *Photobacterium* and *Vibrio* belonging to the Vibrionaceae. Some Iamiaceae, Saprospiraceae and Rhodobacteraceae could not be defined on the genus level.

Eleven phylotypes were identified with both methods. They affiliate to all 4 phyla and were predominantly Rhodobacteraceae of the Alphaproteobacteria and Gammaproteobacteria (Table 1; Figs. 5a, b, 6)

Discussion

In our present study, we investigated the bacterial assemblages of North Sea copepods based on DGGE band sequence data and clone libraries. The use of the 16S rRNA gene as a phylogenetic marker helps to determine the phylogenetic position of bacteria in the evolutionary tree of life independent of cultivability and complexity of the ecosystem (Von Wintzingerode et al. 1997; Hugenholtz et al. 1998). For the first time, the analysis of bacterial assemblages of four copepod key species of Helgoland Roads was carried out over a period of 2 years using culture-independent techniques. We used DGGE for assessing the phylogenetic diversity of the complex microbiome of these four frequently occurring North Sea copepod species. The phylogenetic analysis of the 16S rDNA fragments indicated a relatively low bacterial diversity. In total, 52 different bacterial genera which belong to four bacterial phyla were found to be associated with the four investigated copepod species.

Referring to DGGE analyses, sequences affiliated with the Alphaproteobacteria were predominant in our study. This class was identified as an important group in previous studies on copepods using molecular techniques (Møller et al. 2007; Peter and Sommaruga 2008; Grossart et al. 2009; Tang et al. 2009a, b). In contrast, older studies mostly based on culture-dependent methods found Gammaproteobacteria to be the dominating bacterial class (e.g. Sochard et al. 1979).

Due to methodological limitations of DGGE, only a relatively small part of the 16S rRNA gene can be analysed and used as phylogenetic discriminator. Hence, it is likely that several "species" are combined in some of the OTUs (bands) (Ferrari and Hollibaugh 1999). Additionally, the number of 16S rRNA genes per genome could be a reason for overestimating or underestimating some bacterial groups (Cottrell and Kirchman 2000a). Primer combinations also can cause biases, and with the DGGE primers used in this study, only a few Gammaproteobacteria were found. This was already observed by (Alonso-Sáez et al. 2007) who found that Gammaproteobacteria were not detected in DGGE analysis at all. Primer bias for cloning approaches especially for the Bacteroidetes groups was also discussed previously (Urbanczyk et al. 2007). In our study, Bacteroidetes, an important group of the coastal marine bacterial community (Eilers et al. 2001), were found frequently by DGGE band sequencing as well as in

the clone libraries. In contrast, Cottrell and Kirchman (2000a) stated that Bacteroidetes are underestimated in clone libraries, whereas Alphaproteobacteria are generally overestimated. Hence, care must be taken when deducing the structure of a bacterial community after usage of just one analysis method. We think that in our study, the combination of the two molecular techniques provided further insights into the bacterial assemblages of the investigated marine copepod genera, but currently, we cannot judge whether DGGE or clone libraries represent the real in situ community composition.

Phylogenetic analysis of DGGE band sequences and clone libraries revealed that most of the Alphaproteobacteria phylotypes belong to the Rhodobacterales group (Fig. 5a, b), which is widely distributed and observed frequently in marine environments (Wagner-Döbler and Biebl 2006). Hence, it is not surprising that this group was already identified to be associated with copepods in recent studies (Grossart et al. 2009; Tang et al. 2009a, b; Møller 2007 Møller et al. 2007; Peter and Sommaruga 2008). Rhodobacterales are known to be rapid and successful primary surface colonisers (Porsby et al. 2008; Dang et al. 2008; Jones et al. 2007). The Rhodobacterales phylotypes found in this study clustered with sequences from surface-attached (biotic and abiotic surfaces), but also free-living bacteria. Most of these phylotypes belong to the *Roseobacter* lineage. As ecological generalists, Roseobacter harbour large gene inventories and a remarkable suite of mechanisms by which to obtain carbon and energy (Newton et al. 2010). Members of the *Roseobacter* lineage play an important role for the global carbon and sulphur cycle, for example, and are able to produce dimethylsulfide (DMS) (Wagner-Döbler and Biebl 2006). Dimethylsulfoniumpropionate (DMSP)-consuming bacteria were already found to be associated with the marine calanoid copepod *Acartia tonsa* by Tang et al. (2001). Møller et al. (2007), who first investigated the bacterial community associated with *Calanus helgolandicus* (North Sea) using culture-independent methods (DGGE and subsequent sequencing), found that all of the three obtained bacterial sequences belonged to the Roseobacter lineage. Their phylotype NS17 (DQ839262) clusters with 13 sequences of the present study (Fig. 5b). Hence, it can be assumed that this phylotype is typical for copepods from the North Sea. Overall, 28 phylotypes of the present study are positioned close to the sequences NS16 and NS8 (DQ839261 and DQ839253) (Møller et al. 2007) (Fig. 5a) which again indicates the general association of the Roseobacter lineage with North Sea copepods.

Besides the Rhodobacterales, we also identified phylotypes related to facultative methylotrophic Methylobacteriaceae (Rhizobiales) (Fig. 4).

Finally, we identified phylotypes related to Sphingomonadales (Fig. 4), *Sphingomonas* sp., *Sphingopyxis* sp. and *Erythrobacter* sp. The latter were previously found associated with sea ice, but also with sediment, tunicates and corals. Some *Sphingomonas* sp. are known to express chitinases for usage of chitin as a carbon source (Zhu et al. 2007). Not surprisingly, more phylotypes related to chitin-degrading bacteria were affiliated to the classes of Gammaproteobacteria and Bacteroidetes.

Bacteroidetes have already been found to be associated with copepods (Sochard et al. 1979; Hansen and Bech 1996; Peter and Sommaruga 2008). They are known to be chemoorganotrophic particle colonisers and especially proficient in degrading various polymers such as cellulose, chitin and pectin, but also high-molecular DOM (DeLong et al. 1993; Riemann et al. 2000; Riemann et al. 2006; Kirchman 2002; Cottrell and Kirchman 2000b). In our study, the majority of bacteroidetes phylotypes belong to the Flavobacteriaceae with eight phylotypes related to *Polaribacter* spp. from polar sea ice and sea water (Fig. 3). Three Flavobacteriaceae phylotypes clustered with sequences obtained from Saragossa sea bacterioplankton and from artificial surfaces submerged in sea water. Besides these main phylotypes, single phylotypes were identified within the bacteroidetes, which were previously described for surfaces (Saprospiraceae), sediments or hypersaline microbial mats.

Aside from the Alphaproteobacteria dominating the DGGE analyses, phylotypes of the Gammaproteobacteria were the most abundant in the clone libraries. In many studies, Gammaproteobacteria were identified as the major group associated with marine copepods (Hansen and Bech 1996; Sochard et al. 1979; Heidelberg et al. 2002a). Zooplankton-associated *Vibrio*-populations play an important role in the mineralisation of chitin (Heidelberg et al. 2002a; Belas and Colwell 1982; Huq et al. 1983; Kaneko and Colwell 1975; Tamplin et al. 1990; Bassler et al. 1991; Yu et al. 1991). *Vibrio* spp. comprise a significant portion of the natural bacterial flora of zooplankton with a chitinous exoskeleton (Huq et al. 1983; Heidelberg et al. 2002a; Sochard et al. 1979; Tamplin et al. 1990). The Pseudoalteromonas, however, are also known to possess chitinases. In our study, we found three phylotypes related to *Pseudoalteromonas* spp. (Fig. 6), which were related to sequences from arctic sea water, sea ice, sponges, sediment, chitin enrichments and the nidamental gland and egg capsules of a squid. Hansen and Bech (1996) found *Pseudoalteromonas* spp. associated with copepod intestines and faecal pellets.

Within the Vibrionaceae (Fig. 6), we detected *Aliivibrio* sp. (1 phylotype) and *Vibrio* spp. (6 phylotypes). *Aliivibrio* spp. in the marine environment are often associated with animals; some species are mutualistic symbionts or pathogens of marine animals (Urbanczyk et al. 2007). Six phylotypes in this study cluster with *Vibrio* genera known

as pathogens. *Vibrio alginolyticus* (four phylotypes) causes soft tissue infections and is listed as a human pathogen, whereas *Vibrio splendidus* (two phylotypes) is known to be a fish pathogen (Farmer III and Hickman-Brenner 2006). Some of these species have been associated with mortality in a wide range of marine animals, such as molluscs, fish, shrimps and octopus (Beaz-Hidalgo et al. 2009).

Nine clone sequences clustered with *Photobacterium* spp. *Photobacterium phosphoreum* is a bioluminescent psychrophilic bacterium which is usually found in the intestine, gills and skin of fishes (Farmer III and Hickman-Brenner 2006). *Photobacterium* spp. was also found by (Sochard et al. 1979) associated with the intestine and surface of copepods.

Members of Moraxellaceae build a large group with 18 phylotypes, which fell more precisely into the genus *Psychrobacter*. They clustered with sequences obtained from macroalgae, arctic/antarctic sea ice and sea water, krill and tidal flat sand. *Psychrobacter* species in general do not metabolise complex substrates such as polysaccharides (Bowman 2006). Various *Psychrobacter* species have been found to occasionally cause infections in humans, animals and fish. *Psychrobacter* is considered to be an opportunistic pathogen and generally causes secondary, albeit very serious, infections (Bowman 2006).

Four sequences fell within the Thiothrichaceae, which were previously found associated with sludge, soil, polar pack ice and the gut of bivalves. A novel uncultured *Thiothrix* strain was also found to be endogenous to the marine crustacean *Urothoe poseidonis (*Gillan and Dubilier 2004*).*

Within the Firmicutes (Fig. 2), 5 phylotypes were related to the Bacillales, a single phylotype fell into the Clostridiales. Both groups contain members capable of degrading chitin (Gooday 1990; Vogan et al. 2008). Interestingly, related *Bacillus* spp. phylotypes were already identified by Hansen and Bech (1996) to be associated with the faecal pellets and the gut of copepods.

Within the Actinobacteria (Fig. 2), the 16 phylotypes detected fell into several different families. These bacteria mostly cluster with surface-attached bacteria or seem to originate from sediment, while others are mostly free-living bacteria. Seven of these phylotypes fell into the suborder Micrococcinea. Of these, four sequences belong to the Micrococcaceae. These cluster with sequences from Antarctic ice and soil samples and with a sequence obtained from fish gills. Three phyloptypes were related with Propionibacteriaceae, while another three sequences fell into the Intersporangiaceae, which were found in sediments and associated with corals. Three phylotypes were related to *Microbacterium* spp., known to occur attached to various biotic and abiotic surfaces. Two sequences fell into the Acidimicrobidae and clustered with sponge and North Sea water bacteria, whereas another sequence fell into the Laminaceae and clustered with a sequence from shelf sediment.

Seven Betaproteobacteria phylotypes were found. They were all exclusively related to the Comamonadaceae. The phylotypes cluster with others obtained from sediment and seafloor lavas, but also with faecal samples.

Within the Deltaproteobacteria, we found a single sequence belonging to the genus *Bdellovibrio*. These bacteria have been isolated from a wide range of water systems: estuaries, oceans, rivers, sewage, fish ponds and on biofilms on surfaces. *Bdellovibrios* attack Gram negative bacteria, such as *Vibrio* spp., and they can be regarded as intracellular parasites (Jurkevitch 2006).

In general, the phylogenetic findings can be summarised as follows: (1) Most phylotypes cluster with surface-attached or organism-associated bacteria. (2) The taxonomic groups are in general similar to those already described for marine surfaces or associated with higher marine organisms, but they are often different at higher taxonomic levels (e.g. genera/species). (3) Several phylotypes are closely related to cold-adapted bacteria (e.g. polar ice samples). (4) Several phylotypes are related with chitin-degrading bacteria.

Tang et al. (2009a) postulated that the life strategies of the different copepod genera with regard to feeding strategies play an important role for the composition of the associated bacterial communities. Interestingly, we could not support their hypothesis, since the different copepod genera investigated in this study display different life styles (herbivorous, omnivorous or detrivorous), but did not show significant differences in their bacterial community in respect to DGGE patterns.

Our experimental approach did not allow to distinguish between the bacterial community attached to the exterior of the copepod from that inside the copepod's gut. Although we observed several phylotypes related to *Sulfitobacter* which were previously described for fresh faecal pellets (Jing et al. 2012), the assignment of specific bacterial populations to different body compartments of copepods remains highly speculative. Since the copepod's gut displays environmental conditions which are fundamentally different from those of the surrounding seawater (Tang et al. 2011), it can be assumed that growth of specific bacterial populations is favoured there. Unfortunately, detailed histological studies of copepods with a focus on bacteria colonising the intestine of copepods are still extremely rare (Peter and Sommaruga 2008). Our phylogenetic analysis, covering all closely related sequences so far published in ARB-Silva (Quast et al. 2013), suggests that copepods provide a habitat for bacteria which is different from seawater, but a further differentiation (surface vs. intestine) concerning the bacterial community would be highly speculative.

Copepods do not moult again once they have reached the adult stage (Carman and Dobbs 1997), but until adulthood they shed their carapace after each naupliar and copepodite stage. In this phase of life, the bacterial community is disturbed after each life stage and has to re-colonise the nauplius or copepodite. Thus, the former surface-attached community is lost with the carapace, and colonisation begins anew. These different life stages could be the reason why we found so many primary surface colonisers associated with these copepods. In this context, it might be presumed that the constant supply of chitin could also be an important factor for the development of the bacterial community on the copepod surface. On the other hand, the copepod body may also serve as "a living surface" passively collecting DOC from the environment, while also generating DOC by "sloppy feeding", defecation and excretion. Living surfaces are typically nutrient-rich environments, where organic and inorganic molecules and metabolic by-products accumulate. They often exude different chemical deterrents or cues and are generally morphologically complex (e.g. tissue differentiation). Thus, living surfaces are likely to provide more complex and highly differentiated habitats compared to the pelagic environments.

We assume that the copepod-associated bacteria are mainly opportunistic and benefit from the animal as a "living surface". The colonisation process itself could be most likely explained by the competitive lottery model as already proposed for the colonisation of algal surfaces (Burke et al. 2011b). In this context, we suggest to investigate the link between community structure and function using metagenomic sequence analysis (Burke et al. 2011a) in future studies. From the phylogenetic analysis, it can be emphasis that chitin degradation seems to be a common trait of the bacterial community associated with copepods. Nevertheless, we identified clusters containing only copepod-related bacterial phylotypes, e.g., within the Rhodobacteraceae or within the *Polaribacter* group. Especially, the *Roseobacter* lineage seems to harbour ecologically important copepod colonising phylotypes and should be particularly considered in future studies. Heidelberg et al. (2002a) postulated that the bacterial community associated with zooplankton display seasonal trends, with larger populations during spring and autumn. This hypothesis is in coherence with the study of Huq and Colwell (1996) related to the *Vibrio cholera* epidemics in Bangladesh, which occur biannually, during the spring and fall, and the seasonal cycle of cholera is closely correlated with copepod abundance. In contrast, we were not able to detect any seasonality in the bacterial community of the studied copepods. Interestingly, the bacterial community of the water column of the sampling site Helgoland Roads displays clear seasonality, with well-differentiated successions of different bacterial groups (Gerdts et al. 2004; Alonso-Sáez et al. 2007; Eilers et al. 2001; Teeling et al. 2012; Sapp et al. 2007). By combining these two findings, it can be hypothesised that the "living surface" copepod is selective, attracts specific bacterial populations and provides a niche, which is different from the pelagic environment. Concerning the occurrence of Gammaproteobacteria, care must be taken when interpreting the results due to the obvious bias of the molecular methods. Unfortunately, the primers used for PCR/DGGE seem to discriminate Gammaproteobacteria. Hence, in future studies, different primer sets should be applied (Klindworth et al. 2012). Furthermore, for a comprehensive and "in-depth" analysis of the bacterial community associated with copepods, next-generation sequencing approaches should be applied, as was recently done for the analyses of seawater samples at Helgoland Roads (Teeling et al. 2012). Nevertheless, we never detected human pathogenic *Vibrio* spp. in our waters by cloning. This finding is contradictory to the study of Heidelberg et al. (Heidelberg et al. 2002a) and those studies analysing the *Vibrio* community associated with zooplankton in the tropic waters of Bangladesh (Huq et al. 1983). Since seawater temperature plays a pivotal role in the selection process of bacterial populations, climate change could lead to an increase in human pathogens in the near future also in the temperate European waters and on the "living surface" of North Sea copepods.

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