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Bacterial Community Composition of the Gut Microbiota of *Cylindroiulus fulviceps* (Diplopoda) as Revealed by Molecular Fingerprinting and Cloning

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ABSTRACT. Bacterial clone libraries of the gut microbiota of nurtured and starved *Cylindroiulus fulviceps* specimens displayed the predominance of the phyla Bacteroidetes (55 and 37 %, respectively) and Proteobacteria (40 and 35 %, respectively) and a high similarity to bacteria previously detected in the intestinal tract of termites and beetles, which are known to harbor symbiotic bacteria essential for digestive activity. Bacterial isolates were dominated by Proteobacteria (74 %), followed by members of the phyla Actinobacteria, Firmicutes and Bacteroidetes. PCR–DGGE fingerprints of the gut samples showed that intestinal bacteria were affected by starvation, although the change was not significant.

Abbreviations

DGGE	denaturing gradient gel electrophoresis	OTUs	operational taxonomic units
ICD	intestines of diplopods fed with cow dung	PCO	principal coordinate analysis
ISA	intestines of starved diplopods	RDP	ribosomal database project

Interactions between millipedes and microorganisms are important for soil ecosystem functioning as they affect soil processes such as organic matter decomposition and nutrient cycling. Millipedes thereby play an active role in the comminution and processing of plant litter (Rawlins *et al.* 2006) and affect microbial activity either directly by predation or indirectly by modifying the composition of organic matter (Lavelle *et al.* 1997). Microorganisms are in turn essential operators within the gut of diplopods, as they produce a variety of degrading enzymes (Hopkin and Read 1992). In the alpine region, *Cylindroiulus fulviceps* (Julidae, Diplopoda) is among the key macrodecomposers after the abandonment of pastureland (Seeber *et al.* 2005). This land-use change severely affects alpine vegetation and thus alters the dietary supply of decomposers. Despite this diet shift *C. fulviceps* was found to harbor a stable gut microbiota using DGGE (Knapp *et al.* 2009), which is a valuable tool for rapid and simultaneous fingerprinting analysis of multiple samples. This technique is however biased towards dominant organisms and the sequence information obtained by excising bands from gels is limited (Muyzer *et al.* 1993). In contrast, cloning of genomic DNA is more time-consuming, but provides extended sequence information (Ward *et al.* 1990). The combined use of a fingerprinting method and molecular cloning is thus suitable for gaining a deeper insight into the microbial community composition and has been successfully applied to the intestinal tract of termites and coleopteran species (Schmitt-Wagner *et al.* 2003; Egert *et al*. 2005).

Molecular techniques as well as a classical cultivation approach intended to isolate predominant cultivable bacteria were applied in this study to get more detailed insights into the gut microbial community of *C. fulviceps*. Gut samples of its specimens either fed with cow dung (a preferable food source for decomposer species) or starved for two weeks were analyzed, investigating the phylogenetic affiliation of the gut microbiota and its stability.

MATERIALS AND METHODS

Feeding experiment. Diplopods were hand sampled in autumn 2006 on an alpine pasture at the Kaserstattalm (2000 m a.s.l., Central Alps, Austria; 47°7.529′N 11°17.391′E), immediately transferred to plastic boxes (400 × 300 × 185 mm; 15 L total volume) filled with cow dung as food source and incubated in a climate chamber at 12 °C, which corresponds to the average soil temperature regime on the research site

during the vegetation period. After a 6-week incubation, 10 diplopods were dissected in a sterile workbench; before dissection the diploids were surface sterilized with 70 % ethanol (Knapp *et al.* 2009). For each sample DNA was extracted separately to obtain independent replicates and marked ICD. Five additional *C. fulviceps* specimens were then transferred to a separate box without feed. After two weeks of starvation, their guts were extracted and the samples were labeled ISA.

PCR–DGGE analysis. DNA was extracted from the guts using the PowerSoil DNA Isolation Kit (*MoBio Laboratories*, USA) and amplified with primer set 63f and 1378r as described in Knapp *et al.* (2009). PCR products of the first amplification were used as template for a second PCR using primer pair

338f GC CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG CTC CTA CGG GAG GCA GCA G
and 518r ATT ACC GCG GCT GCT GG (Muyzer *et al.* 1993)

and 30 thermal cycles with an annealing step of ½ min at 56 °C. PCR products were checked by electrophoresis in 1.5 % (*W*/*V*) agarose gels stained by ethidium bromide (10 μg/mL) and quantified using PicoGreen dsDNA quantitation reagent (*Invitrogen*, USA).

DGGE was performed with the Ingeny PhorU2 system (*Ingeny International BV*, The Netherlands). Approximately 60 ng of PCR product was loaded onto 8 % (*W*/*V*) polyacrylamide gels with a denaturing gradient of 40–70 % (100 % denaturant according to 7 mol/L urea plus 40 % formamide in 1× TAE-buffer) and was run for 16 h at 100 V and at 60 °C in 1× TAE-buffer (pH 7.4). The gels were then stained with silver nitrate, photographed and air dried for storage. DGGE banding patterns were normalized and analyzed using the GelCompar II software package, ver. 4.0 (*Applied Maths*, Belgium). Calculation of the pair-wise similarities of fingerprinting patterns was based on the Dice correlation coefficient. Similarity matrices were subjected to PCO using Genstat 11.0 (*VSN International*, UK) and PCO scores were analyzed by one-way ANOVA. Dendrograms were created using the algorithm of Ward.

Bacterial 16S rRNA clone libraries were constructed from the gut sample of specimens fed with cow dung or starved for two weeks. PCR products were generated using the primer pair 8f/1492r (Loy *et al.* 2002) and *Pfu* DNA Polymerase (*Fermentas*, Germany). The PCR program included an initial denaturation step (10 min, 95 °C), 30 thermal cycles (45 s, 95 °C; 45 s, 52 °C; 4 min, 72 °C) and a final extension step (72 °C, 10 min). Amplicons were subsequently purified using the NucleoSpin Extract II Kit (*Machery-Nagel*, Germany). PCR products from the same treatment were pooled and clone libraries were constructed with the TOPO TA Cloning© Kit for Sequencing (*Invitrogen*, USA) according to the manufacturer's protocol. After overnight incubation at 37 °C, 200–250 colonies were picked with sterile toothpicks and regrown overnight at 37 °C.

The inserts were reamplified with the T3/T7 primer pair (TOPO TA Cloning© Kit; *Invitrogen*, USA) by using *E. coli* cells directly as template for the PCR. The program included an initial denaturation (5 min, 94 °C), 30 thermal cycles (50 s, 94 °C; 50 s, 50 °C; 2 min, 72 °C) and a final extension step (72 °C, 10 min). Amplicons were checked for correct length on 1 % (*W*/*V*) agarose gels. Bacterial DNA was amplified with primer set 338fGC-518r and 191 clones from the ICD clone library as well as 170 from the ISA clone library were screened using DGGE (*see above*). Banding patterns were analyzed using GelCompar II and those clones with fingerprinting patterns identical to ≥98 % (this identity level was chosen due to small inter-gel variances) were defined as belonging to a phylotype. Representatives of each phylotype were reamplified with primer set T3/T7, purified and sequenced (*Eurofins MWG Operon*, Germany). Sequence raw data (corresponding to 1200 bp) was checked using CLC DNA Workbench 4.0.1 (*CLCbio*, Denmark) and screened for anomalies using Greengenes (DeSantis *et al.* 2006).

Isolation and identification of culturable microorganisms. Gut samples from the ICD treatment were diluted with 0.95 % (*W*/*V*) NaCl and serial dilutions 10^{-4}–10^{-6} were plated onto ⅓ concentrated nutrient agar plates (Standard I) (in g/L: peptone (meat) 7.8, peptone (casein) 7.8, yeast extract 3, NaCl 6, D-glucose 1, agar 15; pH adjusted to 7) and incubated for 7 d at 14 °C. Frequently occurring colony types were selected according to morphological characteristics such as color, shape and type of growth. Pure cultures were preserved on Standard I slants. DNA from 108 isolates was extracted by a freeze-thawing cycle (30 min at –80 °C, 10 min at 100 °C) or by using the GenElute Bacterial Genomic DNA kit (*Sigma-Aldrich*, USA) and amplified using primer set 338fGC-518r. After screening of isolates using DGGE 43 different OTUs were identified, purified and sequenced, resulting in 23 unique sequences of good quality.

Phylogenetic analysis. Phylogenetic affiliation of the clones and pure cultures was determined using the RDP classifier tool with a confidence level of 80 % (Cole *et al.* 2005). To reveal taxa differing significantly between clone libraries the RDP Library Compare Tool was used, which combines the RDP naïve Bayesian classifier with a statistical test (Wang *et al.* 2007). Sequences were NAST-aligned with reference sequences obtained from the *GenBank* database in Greengenes (DeSantis *et al.* 2006) and phylogenetic trees were constructed based on the neighbor-joining algorithm calculated using the maximum composite likeli-

hood model with the complete deletion option in MEGA4 (Kumar *et al*. 2004). The consistency of the tree was validated by bootstrapping (n = 1000). Chao 1 index as indicator for species richness of the clone libraries was calculated according to Kemp and Aller (2004). The nucleotide sequences have been deposited in *GenBank* under acc.no. FJ798830–FJ798927.

RESULTS

PCR–DGGE analysis of the gut microbiota revealed complex fingerprinting patterns for both sample types, indicating a diverse bacterial community composition within the intestinal tract. Principal coordinate analysis of bacterial fingerprints showed that samples derived from the digestive system of starved diplopods (except ISA5) clustered away from the gut samples of nurtured animals (PCO1: accounting for 17 % variation; PCO2: accounting for 15 % variation) (Fig. 1). ANOVA of PCO scores however did not evidence a significant effect of the food supply (PCO1: $F_{1,13} = 7.31$, $p > 0.01$; PCO2: $F_{1,13} = 5.29$, $p > 0.01$).

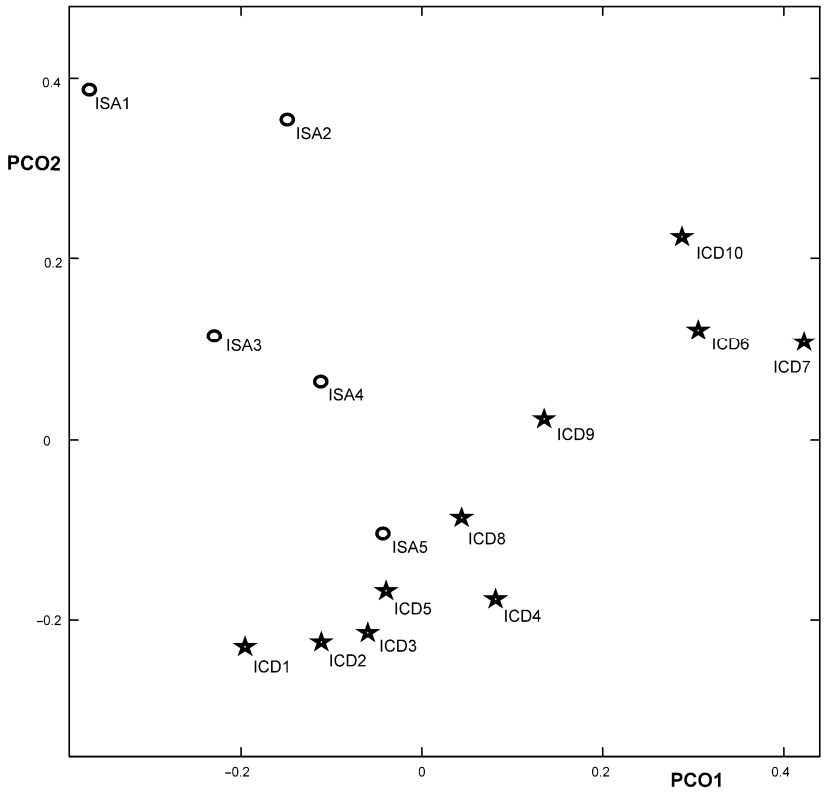

Fig. 1. Ordination plot of the first two canonical variates (PCO2 *vs*. PCO1) of bacterial DGGE fingerprinting patterns after PCO based on DNA extracted from the intestinal tract of *C. fulviceps* specimens ICD (*asterisks*) or ISA (*circles*).

Phylogenetic analysis. From the 361 clones screened on DGGE and used for the classification of phylotypes, 54 and 44 unique phylotypes were identified within the ICD and ISA clone libraries, respectively. Chao 1 index was calculated as indicator for species richness and was found to be low, 68.3 and 52.1 for the clone libraries from ICD and ISA, respectively.

Phylogenetic assignment of the phylotypes showed that Bacteroidetes was the most dominant phylum within both clone libraries, followed by members of the phylum Proteobacteria (α-, β-, γ- and δ-subclass) (Fig. 2). Phylotypes assigned to the class Bacteroidetes belonged to the families Rikenellaceae and Porphyromonadaceae or have not yet been classified. Within both families, clones were frequently affiliated to bacteria already isolated from gut or feces samples of animal and human origin (Fig. 3). Within the Proteobacteria members of the class β-Proteobacteria (30 % of all identified phylotypes) dominated the ISA clone library, whereas γ-Proteobacteria from the family Enterobacteriaceae were most abundant within the ICD clone library (19 % of all identified phylotypes) (Fig. 4). Clones affiliated with the δ-Proteobacteria were

again closely related to bacteria found within the gut of beetle larvae and termites. About 19 % of the phylotypes present in the ICD clone library were attributed to the phylum Firmicutes (class Clostridia), while only few phylotypes of the ISA library were assigned to this phylum.

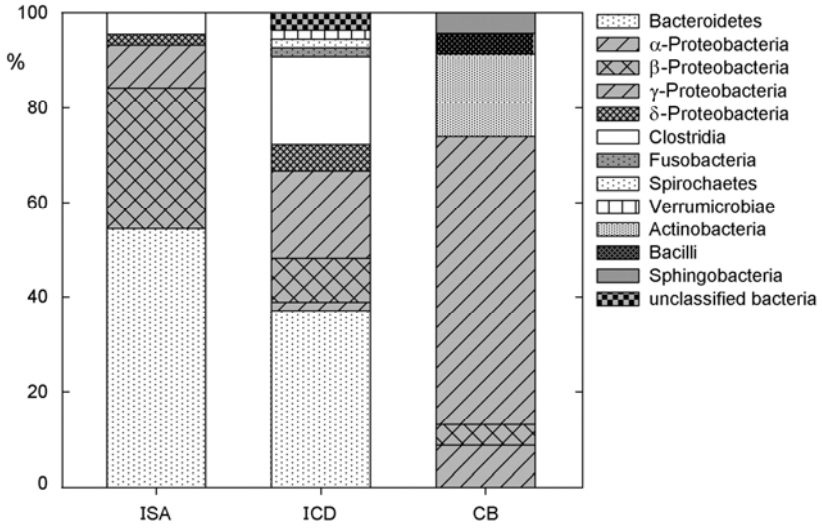

Fig. 2. Phylogenetic affiliation of the clones and the isolates at the class level using the RDP classifier tool with a 80 % confidence level. The *y*-axis represents the abundance (%) of each taxon within gut samples (ICD, ISA) and isolates (CB).

Comparison of the clone libraries using the RDP Library Compare Tool showed that within the β-Proteobacteria the libraries were significantly different on the order-level ($p \leq 0.01$), as members of the order Neisseriales were missing from the ICD clone library. No other significant difference was found between the two libraries.

Identification of culturable microorganisms. Proteobacteria turned out to dominate the bacterial isolates, with >⅔ of all pure cultures being assigned to the α-, β- and γ-subclass of this phylum (Fig. 2). Enterobacteriaceae (γ-Proteobacteria) represented the dominant group and showed a high congruence to sequenced clones (Fig. 4). Members of the Pseudomonadaceae and Xanthomonadaceae (γ-Proteobacteria) were in contrast only found among isolates, whereas no representatives of the δ-Proteobacteria were isolated. Actinobacteria were the second most abundant phylum detected among isolates and members of the phyla Firmicutes (class Bacilli) and Bacteroidetes (class Sphingobacteria) were moreover cultivated.

DISCUSSION

DGGE fingerprints showed that intestinal bacteria were affected by starvation (Figs 1 and 5), although the change was not significant. Microbial communities within the gut of soil diplopods were previously demonstrated to differ from those found in the litter (Byzov *et al.* 1996; Marialigeti *et al.* 1985), but knowledge on their function is limited and it is not clear if these bacteria are specific for the diplopods' gut microbiota (Hopkin and Read 1992).

Cloning and sequencing of the gut microbiota revealed the predominance of Bacteroidetes and Proteobacteria in the intestinal tract, irrespective of the nutritional status (Fig. 2). Numerous phylotypes assigned to the Bacteroidetes were most closely related to clones stemming from the intestinal tract of termites (Hongoh *et al*. 2003; Schmitt-Wagner *et al*. 2003; Yang *et al*. 2005; Nakajima *et al*. 2006) or beetles (Egert *et al*. 2003) (Fig. 3). Together with members of the phylum Firmicutes, Bacteroidetes have been demonstrated to dominate in the gut of termites (Schmitt-Wagner *et al*. 2003; Yang *et al*. 2005; Hongoh *et al*. 2006), inlcuding representatives with fermentative metabolism from the classes Bacteroidetes (phylum Bacteroidetes) and Clostridia (phylum Firmicutes) assumed to contribute to the dissimilation of plant-derived organic materials (Hongoh *et al.* 2006). Some representatives of the class δ-Proteobacteria, which mainly consists of sulfate- and sulfur-reducing bacteria, were also affiliated to sequences originating from the termite (Vu *et al.* 2004; Hongoh *et al*. 2006; Warnecke *et al*. 2007) and beetle gut (FJ374255.1) (Fig. 4). Within the class γ-Proteobacteria, known for its ability to metabolize diverse carbon compounds (Kersters *et al*. 2006), members of the family Enterobacteriaceae have been reported to be abundant in the gut microbiota of invertebrates (Dillon *et al*. 2008; Lee *et al*. 2008) and are supposed to belong to the predominating families within the diplopods' intestinal tract (Byzov *et al*. 1996).

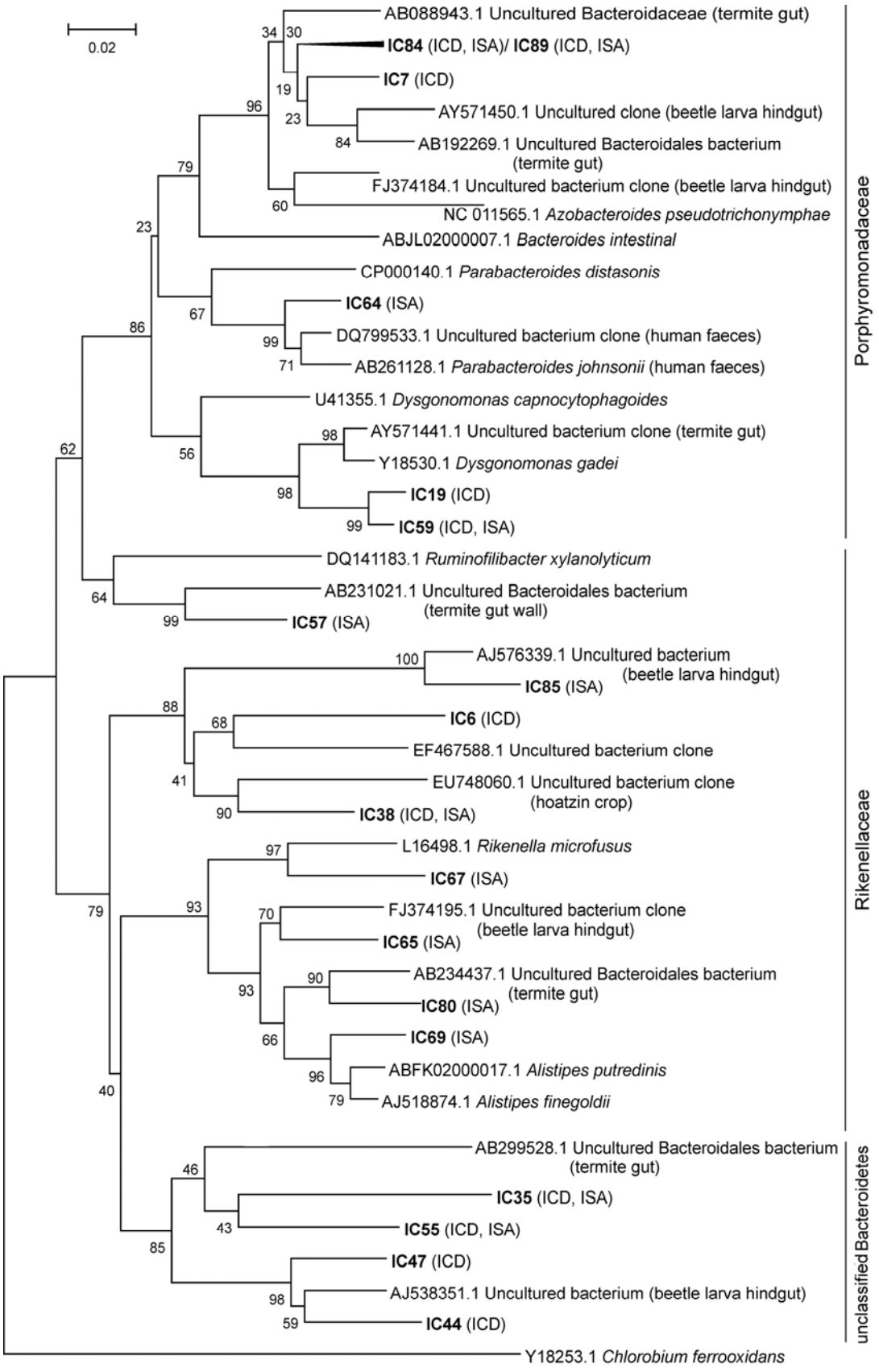

Fig. 3. Neighbor-joining tree of 16S rRNA gene sequences depicting the phylogenetic relationships of bacterial clones from ICD and ISA clone libraries affiliated with the phylum Bacteroidetes; *scale bar* – 2 % sequence divergence, *numbexrs at nodes* – bootstrap values based on 1000 replications; *Chlorobium ferrooxidans* (Chlorobi; Chlorobia) was used as outgroup; **IC** (in **boldface**) – sequences from this paper.

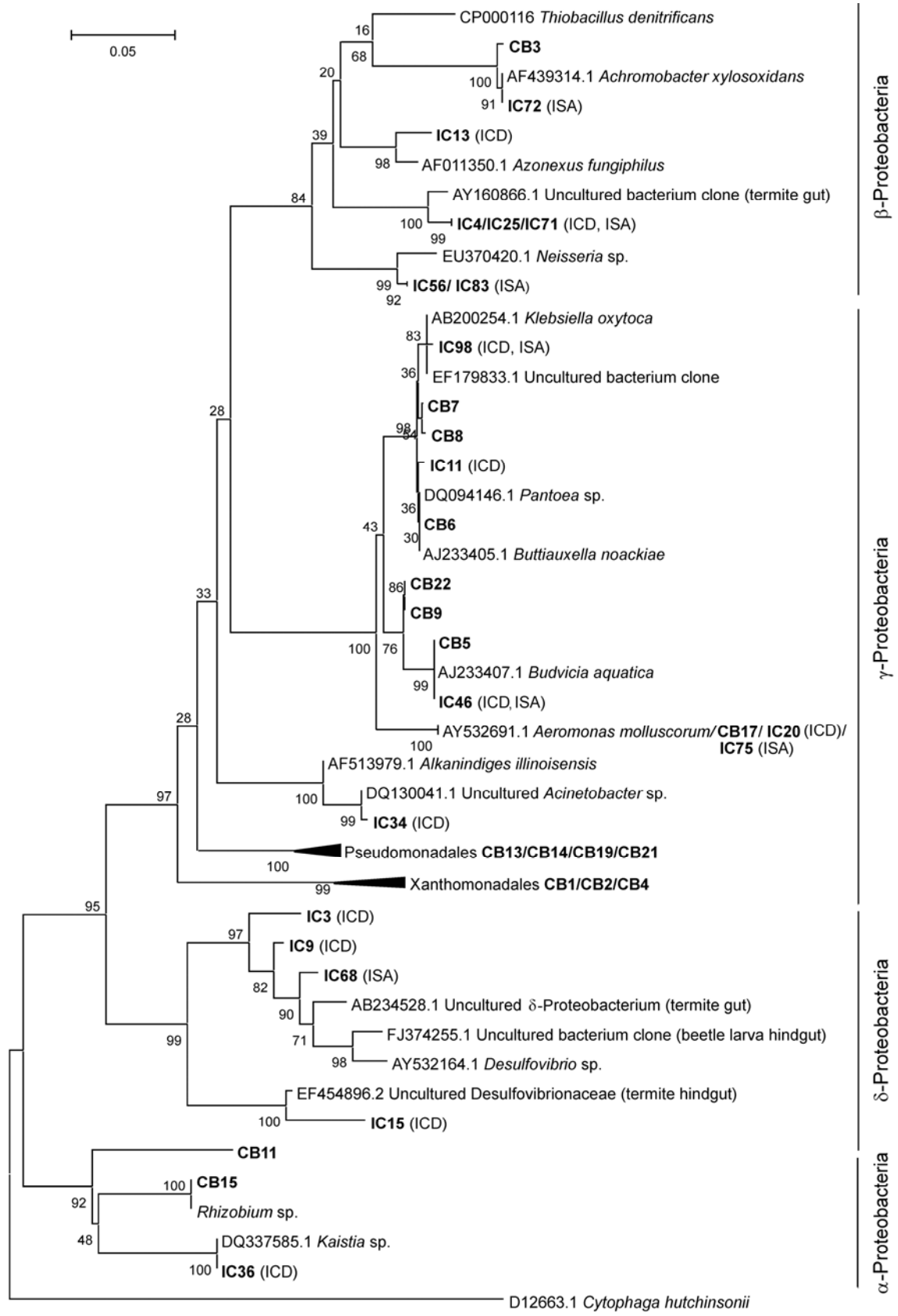

Fig. 4. Neighbor-joining tree of 16S rRNA gene sequences depicting the phylogenetic relationships of bacterial clones isolated from ICD and ISA clone libraries and pure cultures affiliated with the phylum Proteobacteria; *scale bar* – 5 % sequence divergence, *numbers at nodes* – bootstrap values based on 1000 replications; *Cytophaga hutchinsonii* (Bacteroidetes; Sphingobacteria) was used as outgroup; **IC** (clones) and **CB** (pure cultures; both in **boldface**) – sequences from this paper.

Whereas Actinobacteria are well represented in culture collections, they are often under-represented in clone libraries (Hugenholtz *et al.* 1998). Due to the difficult lysis of Actinobacteria cells and their high GC-content, DNA extraction as well as PCR amplification may be hampered (Mühling *et al.* 2008; Feinstein *et al.* 2009). This is why the lack of Actinobacteria within clone libraries may either be ascribed to their low

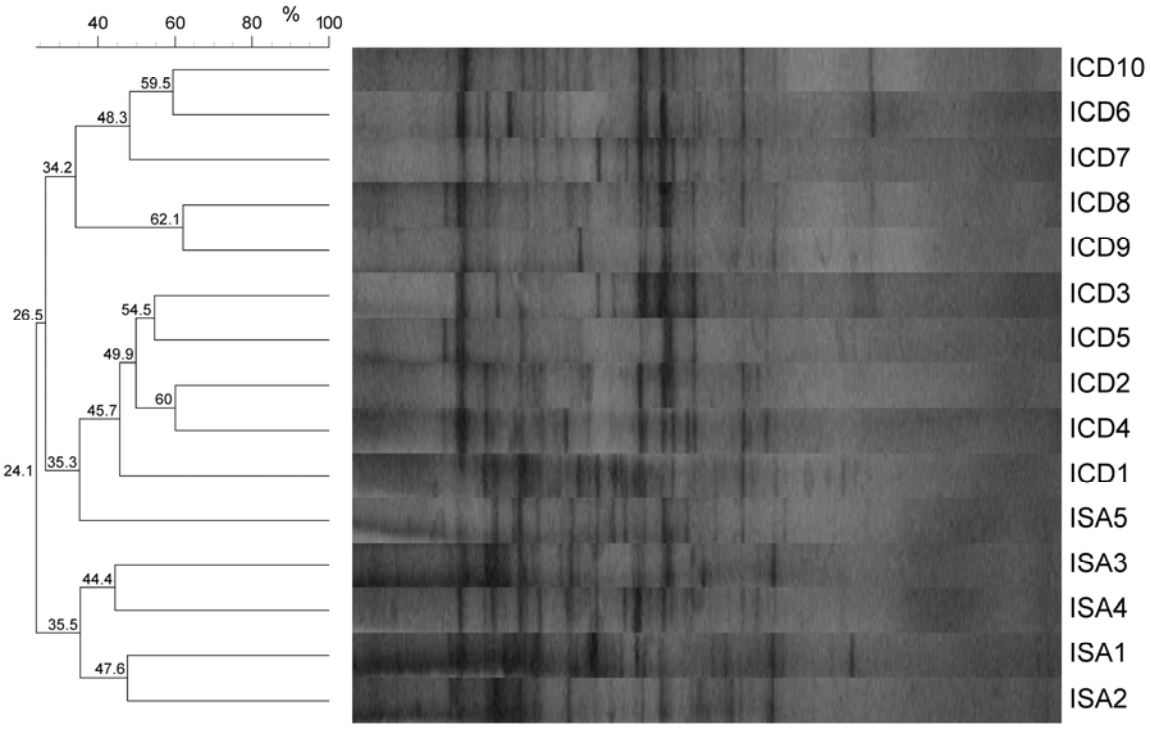

Fig. 5. Cluster analysis of bacterial DGGE fingerprints based on 16S rRNA gene fragments extracted from the intestinal tracts of *C. fulviceps* specimens fed with cow dung (ICD) or starved for two weeks (ISA). Values at the branches of the dendrograms indicate the percentage of similarity, based on the Dice correlation coefficient.

abundance within the habitat or to methodological restrictions. The low abundance of Bacteroidetes, Firmicutes and δ-Proteobacteria among the isolates as compared to the clone libraries may be attributed to their preference for microaerophilic environments previously detected in the gut of diplopods (Byzov *et al.* 2006). Analyzing gut samples derived from *C. fulviceps* using anaerobic cultivation Schrott *et al.* (2009) were indeed able to isolate facultative anaerobic bacteria from the phylum Firmicutes (Bacillales and Clostridiales), but no representatives of the Bacteroidetes and δ-Proteobacteria. We thus underline the relevance of molecular methods for investigating complex habitats, although isolates can be of use for studying metabolic pathways and functional abilities of bacterial species.

Members of the phyla Bacteroidetes and Proteobacteria are abundant in the intestinal tract of *C. fulviceps* and can still be retrieved after starvation. As several bacteria found within the diplopods' guts were closely related to microorganisms previously detected in the intestinal tract of termites and beetles, known to harbor symbiotic bacteria essential for digestive activities, their function in digestion processes should be examined.

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