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**Full Paper**

# Fecal microbiota of a dugong (*Dugong dugong*) in captivity at Toba Aquarium

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**A captive female dugong at Toba Aquarium (Japan) was examined to describe the microbiota of its lower digestive tracts using the molecular-biological technique, a culture-independent method. The phylogenetic analysis of bacterial 16S rRNA genes was conducted for fecal samples, which were taken at 3 different periods. Based on phylogenetic analysis of these sequences, the representatives of six bacterial phyla could be identified: *Actinobacteria* (0.7%), *Bacteroidetes* (15%), *Firmicutes* (83.1%), *Lentisphaerae* (0.1%), *Proteobacteria* (0.1%), and *Verrucomicrobia* (1.0%), suggesting the existence of bacterial species newly found not only in the digestive tract but also in natural field.**

**Key Words**——dugong; eelgrass; fecal microbiota; marine herbivore

## Introduction

Dugong (*Dugong dugong*) is a species of herbivore in the sea, possessing a long colon, 8.6–11.5 times longer than its body size (Kamiya et al., 1979). Like other hindgut mammals the dugongs evolved their body requirement for growth and maintenance by grazing sea grasses (Cork et al., 1998; Erftemeijer et al., 1993; Preen, 1995). Dugongs digest plant cell walls in the cecum and proximal colon (Murray et al., 1977), and a high concentration of short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate, was reported in the cecum and large intestine of a dead dugong. $10^9$–$10^{10}$ colony forming units $g^{-1}$ each of starch-, lactate-, cellobiose-, pectin-, xylan-, and cellulose-utilizing bacteria, sulfate-reducing bacteria and methane-producing bacteria was previously detected in the fecal samples of a captive dugong at Toba Aquarium, showing extensive microbial degradation of cellulolytic and non-cellulolytic components of eelgrass (Goto et al., 2004b). The total SCFA concentration was produced by 10.5 mmol $dl^{-1}$ of 55.7 mol% acetate, 18.0 mol% *n*-butyrate and 15.1 mol% propionate after 36 h incubation. However, the analysis of the microbiota of the digestive tracts is limited in culture-based approaches, especially due to environmental differences between inherent and artificial growth conditions.

The first attempt to keep a dugong (*Dugong dugong*) in captivity was in the United States of America in 1955, when a male dugong was transported from Palau Island, Micronesia, to San Francisco. It survived for only 45 days at the Steinhart Aquarium. Many subsequent attempts have been made by many countries and institutions to keep dugongs. Examples include the Central Marine Fisheries Institute (India), Puket

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Marine Biological Center (Thailand), Yongon Zoo (Myammar), Jaya Ancol Oceanarium (Indonesia) and Cairns Oceanarium (Australia). Most of these attempts to keep dugongs in captivity were short-lived and many difficulties were encountered. From four dugongs brought from the Philippines to Toba Aquarium (Japan) from 1979 to 1987, a 28-year-old male and a 21-year-old female were still alive in 2007 (Toba Aquarium, 1995). Feeding data of these captive dugongs have been continuously recorded since the two dugongs were infants.

In this study a captive female dugong at Toba Aquarium was examined to describe the microbiota of its lower digestive tracts using the molecular- biological technique, a culture-independent method. The phylogenetic analysis of bacterial 16S rRNA genes was conducted for fecal samples, which were taken at 3 different periods.

## Materials and Methods

*Animal and sampling.* Fecal samples were collected from a captive female dugong in Toba Aquarium (Age: 21 years old in 2007; body weight: 345 kg), which was kept and fed eelgrass (*Zostera marina*) under the control conditions (water temperature at 28°C and natural lighting) as described previously (Goto et al., 2004a). The samples were taken at 3 different periods, namely May 2000, October 2004, and August 2005, by applying fingertip pressure to the urogenital area of the female dugong floating on its back without pool water or any other contaminant. The sample in a 20 ml polypropylene container was stored at −80°C until further processing.

*Extraction and purification of DNA.* A 0.5 g of sample was randomly collected from inner and outer portions of a couple pieces of fecal samples, and the DNA purification of the sample collected in the year 2000 was performed according to a chemical procedure of the QIAamp DNA Stool Mini Kit (Qiagen, Germany) (sample A in Table 1) and that of the samples collected in 2000, 2004, and 2005 (sample B, C, D, respectively, in Table 1) was by a physical procedure described by Godon et al. (1997).

According to the method of Godon et al. (1997), a solid portion of the fecal sample was collected by centrifugation and suspended in 500 µl of 4 M guanidine thiocyanate-0.1 M Tris (pH 7.5) and 75 µl of 10% N-lauroyl sarcosine. Two hundred fifty microliters of this material was transferred to a 2-ml screw-cap polypropylene microtube, which contained 500 µl of 5% N-lauroyl sarcosine-0.1 M phosphate buffer (pH 8.0) and 0.38 g of 0.1 mm diameter silica beads previously sterilized. This tube was then incubated at 70°C for 1 h and shaken 2 times at maximum speed for 45 s each, using a Fast Prep FP100A Instrument (Qbiogene). Polyvinyl polypyrrolidone (15 mg) was added to the tube, which was vortexed and centrifuged for 3 min at 13,000×*g*. After recovery of the supernatant, the pellet was washed with 500 µl of TENP (50 mM Tris [pH 8], 20 mM EDTA [pH 8], 100 mM NaCl, 1% polyvinyl polypyrrolidone) and centrifuged for 3 min at 13,000×*g*, and the new supernatant was added to the first supernatant. The washing step was repeated three times. The pooled supernatants (about 2 ml) were split between two 2-ml tubes. Nucleic acids were precipitated by the addition of 1 volume of isopropanol for 10 min at room temperature and centrifuged for 3 min at 13,000×*g*. Pellets were resuspended and pooled in 450 µl of 100 mM phosphate buffer (pH 8), and 50 µl of 5 M potassium acetate. The tube was placed on ice for 90 min and centrifuged at 13,000×*g* for 30 min. The supernatant was transferred to a new tube containing 20 µg of RNase (1 mg/ml) and incubated at 37°C for 30 min. Phenol-chloroform-isoamyl-alcohol (25 : 24 : 1) was added, mixed for 5 min, and centrifuged for 5 min at 13,000×*g*. Nucleic acids were precipitated by the addition of 50 µl of 3 M sodium acetate and 1 ml of absolute ethanol to the supernatant. The tube was incubated for 5 min at room temperature, and the nucleic acids were recovered by centrifugation at 15,000×*g* for 5 min. The DNA pellet was finally washed with 70% ethanol, dried, and resuspended in 100 µl of TE buffer. Recovered DNA was purified using AutoSepTM G-50 (Amersham Pharmacia Biotech).

*16S rDNA amplification, cloning and restriction fragment length polymorphism.* Two universal primers, 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′) were used in PCR to amplify the 16S rRNA coding region (rDNA). The amplification reaction was performed using the following program: 20 cycles consisting of 96°C for 10 s, 53°C for 10 s., 72°C for 4 min, and a final extension period of 72°C for 10 min. PCR products were cloned into the plasmid vector pCR2.1 and competent *Escherichia coli* INVα were transformed using a TA-cloning kit (Invitrogen), according to manufacturer's instructions.

The inserted DNA region was amplified by the PCR with the set of primers. PCR products were digested with *Hae*III restriction endonuclease and grouped by the pattern of band on an acrylamide gel electrophoresis.

*Sequence analysis and nucleotide sequence accession number.* The representative of the groups was sequenced using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences). The nucleotide products and their sequences were analyzed on a DNA analyzer system (ABI PRISM 3100, Applied Biosystems) and with BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). All near-full-length sequences were tested for possible chimeric structures. Chimeras were detected by the RDP analysis service Check_Chimera (Larsen et al., 1993).

Clone 16S rDNA sequences, their closest relatives identified from database searches, and appropriate type strain sequences were aligned with Clustal W (Thompson et al., 1994) and phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with Clustal W. The near-full-length 16S rDNA sequences have been deposited in the DDBJ database under accession numbers AB218288 to AB218350 and AB264058 to AB264084.

## Results

A total of 709 16S rRNA genes, nearly complete sequences averaging 1,500 bp, were obtained from the dugong's fecal sample. Only one of the sequences was identical with *Catabacter hongkongenesis* HKU16, indicating that the study was far from defining the full extent of the taxonomic diversity contained within the libraries (Table 1).

Based on phylogenetic analysis of these sequences, the representatives of six bacterial phyla could be identified: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Lentisphaerae*, *Proteobacteria* (delta subclass) and *Verrucomicrobia*. The vast majority (83.1%) of the sequences were affiliated with *Firmicutes*. Except for one, none of the sequences matched any 16S rRNA gene sequence that was derived from a previously cultivated species, and therefore the majority of the sequences that were obtained in this study represented microbial species previously uncharacterized. In many cases, there were nearest matched clones to the 16S rRNA genes of uncultured bacteria from pig and human digestive tracts detected previously. Figures 1 to 4 show the phylogenetic deductions among one of the taxonomic units affiliated with *Clostridium*, which was divided into 19 major clusters (Collins et al., 1994). The majority (386 clones) of our sequences was associated with these clusters, primarily with Subcluster XIVa (186 clones), which includes *Clostridium polysaccharolyticum* and *Eubacterium xylanophilum*. Similarly, large numbers of sequences were associated with Cluster IV (85 clones) and Cluster I (68 clones). Small numbers of the sequences were associated with Cluster III (11 clones), Cluster IX (20 clones) and Cluster XI (16 clones).

The similarity in the patterns of compositional percentage of the major three groups, *Mollicutes* (34–37%), and *Clostridium* rRNA clusters I (12–19%) and IV (14–28%) was observed between the above-mentioned commercial kit and procedures described by Godon et al. (1997) for bacterial DNA extraction, as sampled in May 2000 (Sample A and B, Table 1).

Variations in patterns of the compositional percentage of clones detected were observed between fecal samples of May 2000 and October 2004 or August 2005, showing a drastic shift of major groups from *Mollicutes* and *Clostridium* rRNA Clusters I and IV in the year 2000 to *Clostridium* rRNA Subclusters XIVa and *Cytophaga-Flexibacter-Bacteroides* in the years 2004 and 2005.

## Discussion

The first attempt to describe the bacterial biota in fecal contents of the dugong was carried out using 16S rDNA library analysis and suggested the existence of bacterial species newly found not only in the digestive tract but also in natural field.

The nearest species from BLAST search were gastrointestinal bacteria from pigs (Leser et al., 2002), humans (Derrien et al., 2004; Dewhirst et al., 2001), mice (Dewhirst et al., 1999; Ley et al., 2005; Salzman et al., 2002), and wild herbivores (Nelson et al., 2003). They were also the bacteria in the rumen and intestinal digestive tract of dairy cattle (Tajima et al., 1999), although it was of low similarity.

In general, the bacterial biota in the digestive tracts of terrestrial and marine herbivores can vary depending the digestion mode and system, which is represented by fore stomach fermentation and hindgut fermentation. As the library of fecal clones found in this study was compared with that of the microbiota in di-

Table 1. Similarity values and frequencies of isolated clones from dugong feces.

| Represent No. | Accession No. | Phylum | Class (Subclass) | Family | Nearest species (Accession No.) | Homology (%) | Clones A 2000/5 | Clones B 2000/5 | Clones C 2004/10 | Clones D 2005/8 | Total clones |
|---|---|---|---|---|---|---|---|---|---|---|---|
| dgC-118 | AB218329 | *Actinobacteria* | *Actinobacteria* | *Coriobacteriaceae* | *Atopobium parvulum* ATCC22793 (AF292372) | 93.3 | | | 1 | 2 | 3 |
| dgD-191 | AB264083 | | | | Uncultured bacterium clone p-275-o5 (AF371713) | 90.3 | | | | 2 | 2 |
| dgC-30 | AB218330 | *Bacteroidetes* | *Bacteroidetes* | *Bacteroidaceae* | Bacterium mpn-isolate group 5 (AF357553) | 99.7 | | | 1 | | 1 |
| dgD-135 | AB264075 | | | | | 99.5 | | | | 1 | 1 |
| dgC-78 | AB218331 | | | | *Parabacteroides distasonis* ATCC8503 (M86695) | 98.4 | | | 2 | 2 | 4 |
| dgB-5 | AB218313 | | | | *Bacteroides* sp. AR20 (AF139524) | 99.7 | | 8 | 11 | 1 | 20 |
| dgA-180 | AB218288 | | | | *Bacteroides* sp. ASF519 (AF157056) | 96.5 | 1 | | | 2 | 3 |
| dgB-89 | AB218314 | | | | *Bacteroides* sp. CIP103040 (AF133539) | 99.0 | | 1 | | | 1 |
| dgC-101 | AB218332 | | | | *Bacteroides* sp. BV-1 (X89217) | 98.0 | | | 3 | | 3 |
| dgD-156 | AB264079 | | | | *Bacteroides uniformis* mat-344 (AB215084) | 93.1 | | | | 3 | 3 |
| dgD-138 | AB264076 | | | | Uncultured bacterium clone C686 (AY985676) | 99.9 | | | | 2 | 2 |
| dgC-28 | AB218333 | | | | Uncultured bacterium clone C706 (AY916343) | 99.6 | | | 1 | 1 | 2 |
| dgA-11 | AB218289 | | | | Uncultured bacterium clone FE1 (AY553946) | 90.9 | 1 | | | | 1 |
| dgC-176 | AB218334 | | | | Uncultured bacterium clone M516 (AY916163) | 98.4 | | | 1 | | 1 |
| dgD-75 | AB264070 | | | *Prevotellaceae* | Uncultured bacterium clone C5-79 (DQ113691) | 93.0 | | | | 1 | 1 |
| dgC-158 | AB264058 | | | | Uncultured bacterium clone MH03 (AY982206) | 93.2 | | | 12 | | 12 |
| dgA-52 | AB218301 | | | | Uncultured bacterium clone p-317-a3 (AF371895) | 96.1 | 4 | 6 | 12 | 19 | 41 |
| dgD-128 | AB264074 | | | | | 96.0 | | | | 1 | 1 |
| dgD-23 | AB264064 | | | | | 95.8 | | | | 1 | 1 |
| dgD-17 | AB264062 | | | | Uncultured rumen bacterium clone S7 (DQ256287) | 90.8 | | | | 4 | 4 |
| dgC-96 | AB218335 | | | *Rikenellaceae* | *Alistipes finegoldii* ANH 2437 (AJ518874) | 99.2 | | | 2 | 1 | 3 |
| dgD-59 | AB264069 | | unknown | | Uncultured bacterium clone C272 (AY985351) | 95.1 | | | | 1 | 1 |
| dgB-66 | AB218316 | *Firmicutes* | *Bacilli* | *Acidaminococcaceae* | Uncultured bacterium clone p-286-a3 (AF371705) | 92.2 | | 1 | 7 | 12 | 20 |
| dgA-92 | AB218290 | | | *Streptococcaceae* | *Lactococcus lactis* subsp. *lactis* IL1403 (NC 002662) | 99.8 | 2 | | | | 2 |
| dgC-135 | AB218336 | | | unknown | Uncultured bacterium clone IIIA-3 (AJ488093) | 95.1 | | | 3 | | 3 |
| dgA-6 | AB218291 | | | | | 95.0 | 1 | | | | 1 |
| dgB-82 | AB218315 | | | | | 94.8 | | 6 | 1 | 5 | 12 |
| dgB-113 | AB218317 | | *Clostridia* | *Clostridiaceae* | *Clostridium butyricum* NCIMB8082 (X68178) | 98.5 | | 14 | 2 | | 16 |
| dgD-10 | AB264061 | | | | *Clostridium cellulosi* AS1.1777 (L09177) | 92.8 | | | | 2 | 2 |
| dgA-143 | AB218292 | | | | *Clostridium longisporum* DSM 8431 (X76164) | 98.6 | 4 | 4 | 4 | 5 | 17 |
| dgD-151 | AB264078 | | | | *Clostridium subterminale* DSM 6970 (AB294137) | 97.3 | | | | 1 | 1 |
| dgB-84 | AB218319 | | | | | 97.1 | | 9 | 4 | | 13 |

Table 1. Continued.

| Represent No. | Accession No. | Phylum | Class (Subclass) | Family | Nearest species (Accession No.) | Homology (%) | Clones A 2000/5 | Clones B 2000/5 | Clones C 2004/10 | Clones D 2005/8 | Total clones |
|---|---|---|---|---|---|---|---|---|---|---|---|
| dgA-13 | AB218293 | | | | | 96.8 | 5 | | | | 5 |
| dgA-121 | AB218294 | | | | | 96.6 | 6 | | | | 6 |
| dgA-7 | AB218295 | | | | | 96.2 | 7 | | | | 7 |
| dgC-58 | AB218337 | | | | | 95.8 | | | 1 | | 1 |
| dgD-19 | AB264063 | | | | *Clostridium symbiosum* ATCC14940 (M59112) | 98.2 | | | | 2 | 2 |
| dgD-25 | AB264065 | | | | *Faecalibacterium prausnitzii* clone 1-84 (AY169429) | 92.0 | | | | 2 | 2 |
| dgB-33 | AB218323 | | | | Swine manure pit bacterium PPC78A (AY542483) | 92.8 | | 1 | | | 1 |
| dgB-159 | AB218318 | | | | | 92.6 | | 1 | | | 1 |
| dgC-113 | AB218338 | | | | Uncultured bacterium clone C288 (DQ326314) | 94.3 | | | 2 | | 2 |
| dgC-20 | AB218350 | | | | Uncultured bacterium clone C437 (AY985477) | 95.6 | | | 1 | | 1 |
| dgB-96 | AB218322 | | | | | 95.4 | | 1 | | | 1 |
| dgC-61 | AB218339 | | | | Uncultured bacterium clone ELAND_15 (AY858495) | 99.8 | | | 1 | 2 | 3 |
| dgA-54 | AB218296 | | | | Uncultured bacterium clone ELAND_41 (AY858503) | 99.4 | 3 | 2 | 1 | 2 | 8 |
| dgA-42 | AB218297 | | | | Uncultured bacterium clone HuAC05 (AY684393) | 99.0 | 1 | | | | 1 |
| dgD-96 | AB264071 | | | | Uncultured bacterium clone p-1894-s962-3 (AF371638) | 93.1 | | | | 1 | 1 |
| dgD-50 | AB264067 | | | | Uncultured bacterium clone p-211-o5 (AF371674) | 95.2 | | | | 2 | 2 |
| dgA-181 | AB218298 | | | | Uncultured bacterium clone p-30-a5 (AF371665) | 95.6 | 1 | | | | 1 |
| dgA-71 | AB218299 | | | | | 95.4 | 1 | 9 | 62 | 63 | 135 |
| dgA-73 | AB218300 | | | | | 95.3 | 1 | | | | 1 |
| dgB-70 | AB218327 | | | | Uncultured bacterium clone p-4157-6Wa5 (AF371760) | 99.9 | | 1 | | | 1 |
| dgA-101 | AB218308 | | | | Uncultured bacterium clone p-5459-2Wb5 (AF371947) | 99.7 | 1 | | 3 | | 4 |
| dgA-67 | AB218303 | | | | Uncultured bacterium clone p-594-a5 (AF371763) | 94.5 | 1 | | | 2 | 3 |
| dgC-140 | AB218342 | | | | Unidentified eubacterium from anoxic bulk soil BSV34 (AJ229194) | 93.0 | | | 1 | 1 | 2 |
| dgB-121 | AB218324 | | | *Eubacteriaceae* | *Eubacterium contortum* ATCC25540 (L34615) | 96.5 | | 1 | | | 1 |
| dgA-38 | AB218304 | | | | *Eubacterium* sp. oral strain A35MT (AF287761) | 93.7 | 4 | 2 | 5 | 1 | 12 |
| dgA-3 | AB218305 | | | | *Mogibacterium neglectum* ATCC700924 (AB037875) | 91.4 | 2 | | | | 2 |
| dgB-141 | AB218325 | | | | Uncultured *Eubacteriaceae* bacterium Rs-E61 (AB088987) | 94.7 | | 2 | | | 2 |
| dgD-107 | AB264072 | | | *Lachnospiraceae* | *Lachnospira pectinoschiza* clone 1-10 (AY169414) | 94.7 | | | | 1 | 1 |
| dgD-52 | AB264068 | | | | Uncultured bacterium clone p-2418-55G5 (AF371611) | 95.5 | | | | 4 | 4 |
| dgC-170 | AB264059 | | | | | 95.4 | | | 3 | | 3 |
| dgA-35 | AB218306 | | | | Uncultured bacterium clone p-4176-6Wa5 (AF371772) | 91.3 | 6 | 19 | | | 25 |

Table 1. Continued.

| Represent No. | Accession No. | Phylum | Class (Subclass) | Family | Nearest species (Accession No.) | Homology (%) | Clones A 2000/5 | Clones B 2000/5 | Clones C 2004/10 | Clones D 2005/8 | Total clones |
|---|---|---|---|---|---|---|---|---|---|---|---|
| dgA-72 | AB218307 | | | | | 91.2 | 42 | | | | 42 |
| dgB-138 | AB218328 | | | | | 91.0 | | 1 | | | 1 |
| dgA-83 | AB218309 | | | | Uncultured bacterium clone p-956-s962-5 (AF371797) | 96.8 | 13 | | | | 13 |
| dgC-130 | AB218346 | | | | | 96.1 | | | 8 | 12 | 20 |
| dgC-85 | AB218344 | | | | Uncultured bacterium clone Thompsons100 (AY854298) | 98.1 | | | 5 | | 5 |
| dgD-165 | AB264080 | | | unknown | *Catabacter hongkongensis* HKU16 (AY574991) | 100.0 | | | | 1 | 1 |
| dgB-20 | AB218320 | | | | *Clostridium aminophilum* F (L04165) | 89.7 | | 2 | | | 2 |
| dgD-197 | AB264084 | | | | *Clostridium lentocellum* DSM 5427 (X76162) | 92.5 | | | | 1 | 1 |
| dgD-32 | AB264066 | | | | Uncultured bacterium clone D-74 (DQ116011) | 92.2 | | | | 1 | 1 |
| dgD-7 | AB264060 | | | | Uncultured bacterium clone M3_e02 (DQ015386) | 89.0 | | | | 1 | 1 |
| dgC-100 | AB218340 | | | | Uncultured bacterium clone p-1321-a5 (AF371673) | 94.3 | | | 17 | 4 | 21 |
| dgB-67 | AB218326 | | | | Uncultured bacterium clone p-1977-s962-5 (AF371543) | 95.0 | | 3 | 2 | 4 | 9 |
| dgC-129 | AB218345 | | | | | 94.6 | | | 1 | | 1 |
| dgC-172 | AB218343 | | | | Uncultured bacterium clone p-2001-s959-5 (AF371658) | 95.0 | | | 1 | 1 | 2 |
| dgA-80 | AB218302 | | | | Uncultured bacterium clone p-5291-2Wa3 (AF371940) | 94.7 | 2 | | | | 2 |
| dgC-42 | AB218341 | | | | Uncultured eubacterium WCHB1-54 (AF050582) | 92.8 | | | 1 | | 1 |
| dgD-185 | AB264082 | | | | Uncultured rumen bacterium clone U29-A06 (AB185744) | 93.4 | | | | 1 | 1 |
| dgD-113 | AB264073 | | | | Unidentified rumen bacterium RC5 (AF001698) | 91.8 | | | | 1 | 1 |
| dgA-44 | AB218310 | | | | *Sedimentibacter hongkongensis* KI (AY571338) | 93.7 | 4 | 4 | | 4 | 12 |
| dgA-88 | AB218311 | | *Mollicutes* | *Erysipelotrichaceae* | *Bulleidia moorei* AHP 13983 (AY044915) | 91.8 | 64 | 49 | 1 | 3 | 117 |
| dgC-125 | AB218347 | | | unknown | *Allobaculum stercoricanis* DSM 13632T (AJ417075) | 93.5 | | | 1 | 2 | 3 |
| dgB-10 | AB218321 | | | | Unidentified eubacterium clone vadinHA31 (U81729) | 89.5 | | 4 | | | 4 |
| dgC-74 | AB218348 | *Lentisphaerae* | unknown | | Unidentified eubacterium clone vadinHB65 (U81755) | 96.9 | | | 1 | | 1 |
| dgC-139 | AB218349 | *Proteobacteria* | *Deltapro-teobacteria* | *Desulfovibri-onaceae* | Uncultured bacterium clone ELAND_96 (AY858543) | 99.6 | | | 1 | | 1 |
| dgD-149 | AB264077 | *Verrucomicrobia* | *Verrucomi-crobiae* | *Verrucomi-crobiaceae* | *Akkermansia muciniphila* Muc (AY271254) | 99.7 | | | | 3 | 3 |
| dgD-170 | AB264081 | | | | Uncultured bacterium clone HuCA18 (AJ408970) | 95.0 | | | | 1 | 1 |
| dgA-65 | AB218312 | | | | Uncultured bacterium clone L10-6 (AJ400275) | 99.2 | 2 | 1 | | | 3 |
| Total clones | | | | | | | 179 | 152 | 186 | 192 | 709 |

A: Total DNA was prepared with QIAamp DNA Stool Mini Kit; B, C, and D were prepared with a method described by Godon et al. (1997).

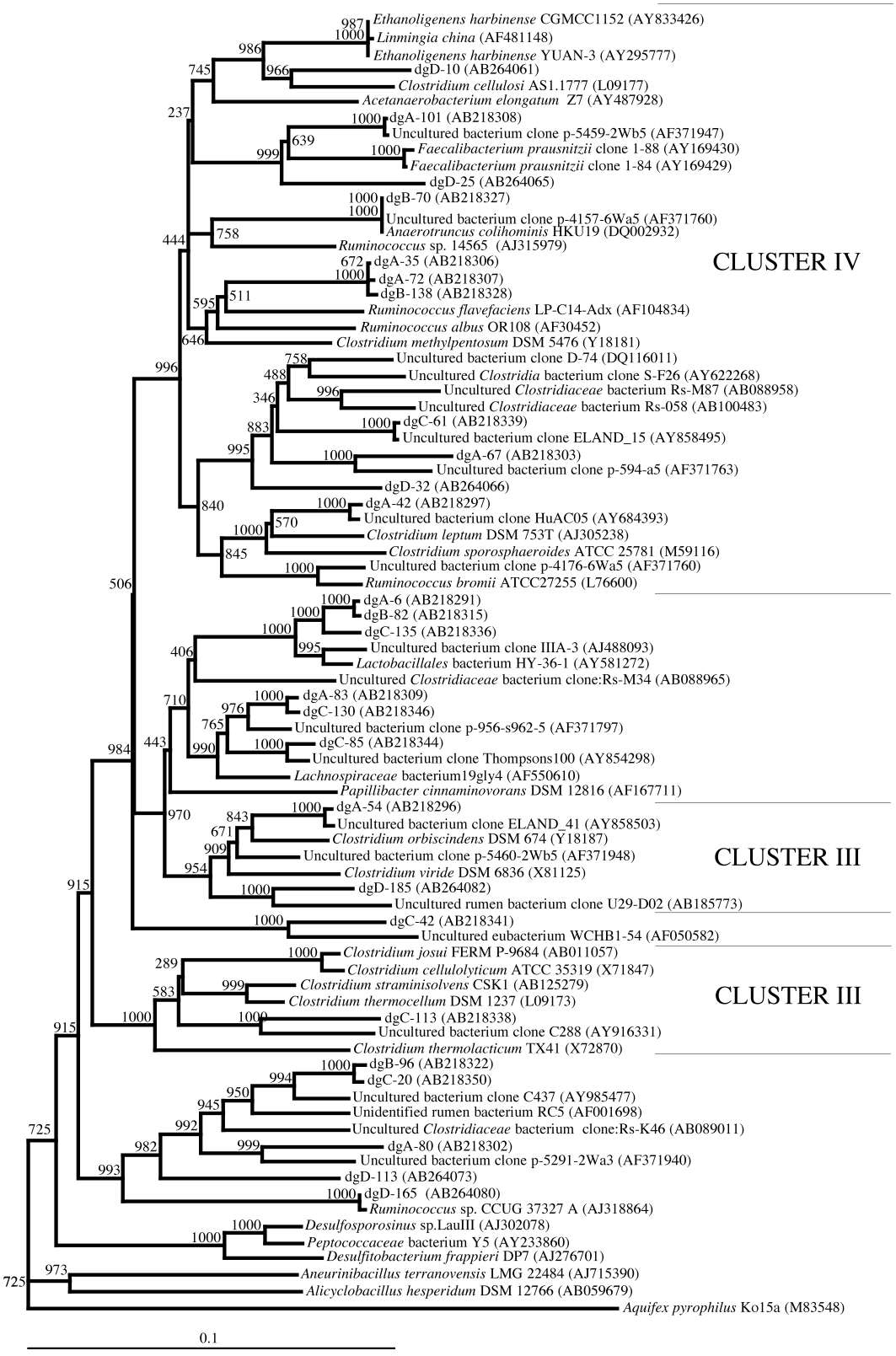


Fig. 1. Phylogenetic tree of cloned 16S rRNA genes and their closest relatives-1.

The tree was created using the neighbor-joining method with Clustal W. The numbers on the tree indicate bootstrap values for the branching points. The sequence data for closest relatives were obtained from the DNA databases (Genbank/EMBL/DDBJ). Clone names, dgA, dgB, dgC, and dgD were sequenced in this study (See in Table 1).

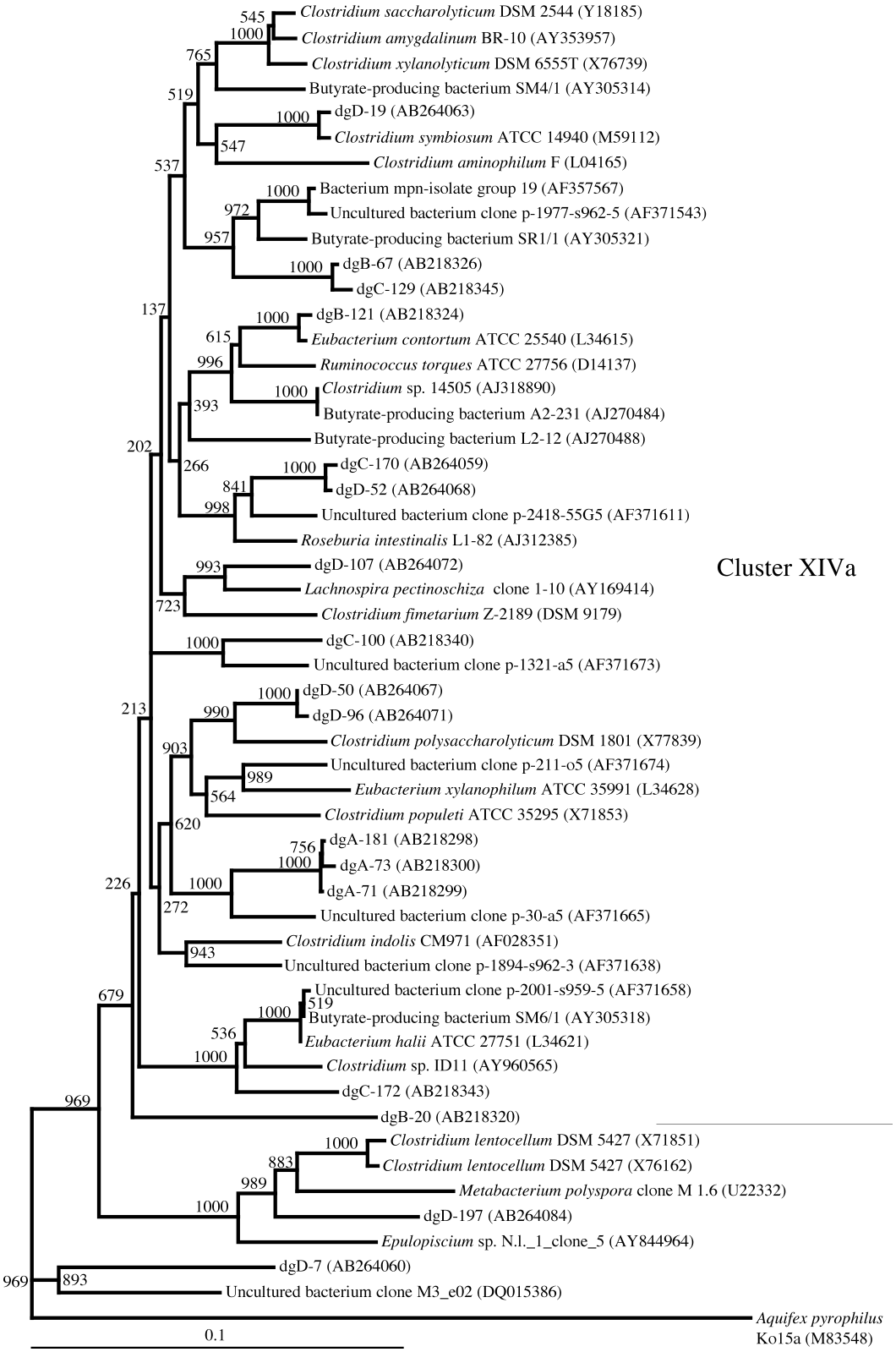


Fig. 2. Phylogenetic tree of cloned 16S rRNA genes and their closest relatives-2. See the legend to Fig. 1 for explanation.

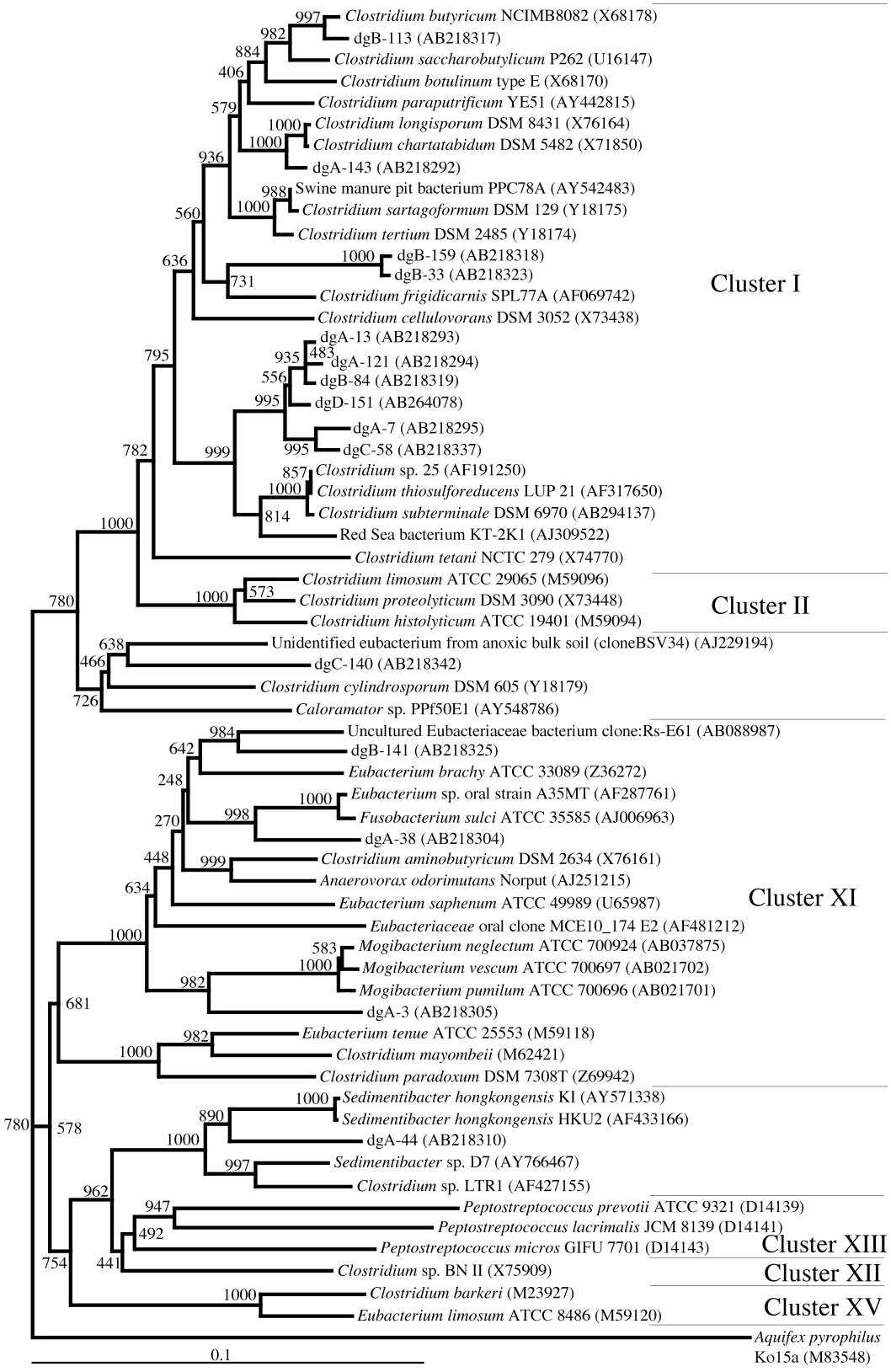


Fig. 3. Phylogenetic tree of cloned 16S rRNA genes and their closest relatives-3. See the legend to Fig. 1 for explanation.

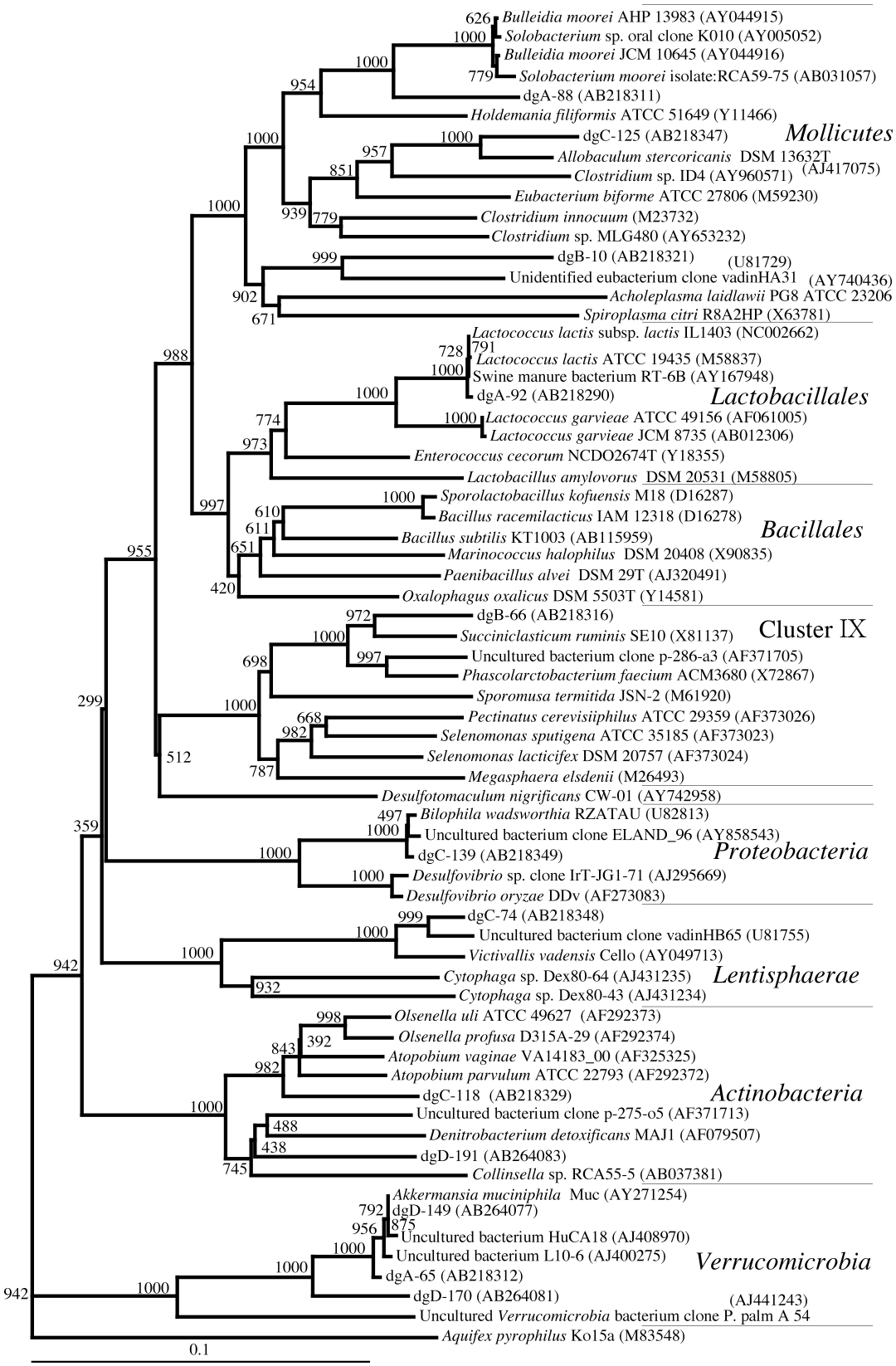


Fig. 4. Phylogenetic tree of cloned 16S rRNA genes and their closest relatives-4.
See the legend to Fig. 1 for explanation.

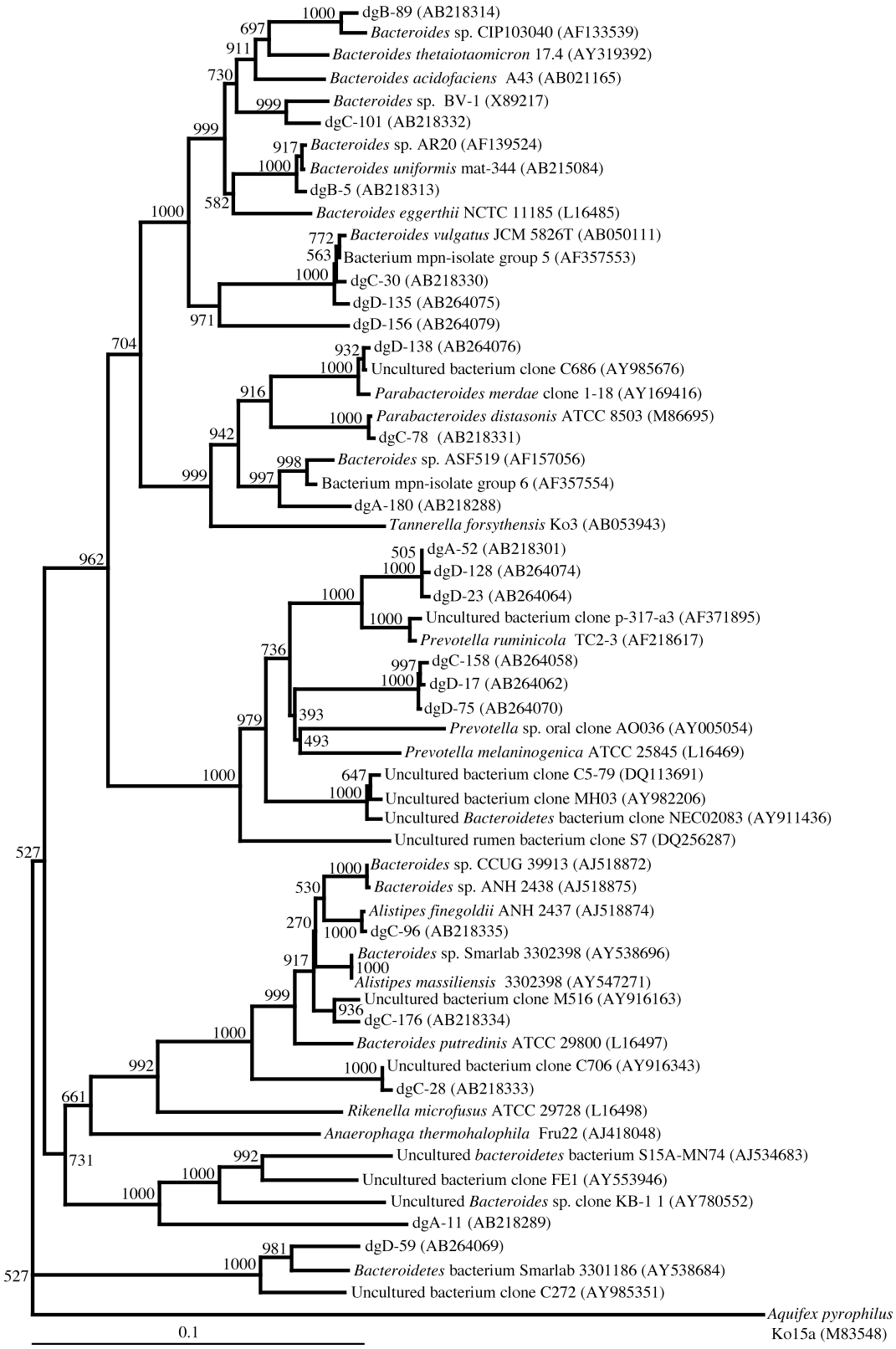


Fig. 5. Phylogenetic tree of cloned 16S rRNA genes and their closest relatives-5. See the legend to Fig. 1 for explanation.

gestive tract of dairy cattle, a fore stomach fermenter, there were differences in the compositional percentage of the clones between the dugong's fecal contents and contents of the rumen and feces of dairy cattle. The composition of the *Firmicutes* phylum was higher for the dugong's fecal contents (83.1%) as compared to those of cattle's ruminal contents (52.4%), while the lower composition of *Cytophaga-Flexibacter-Bacteroides* of the dugong (15.0%) was observed as compared to the cattle (38.1%, Tajima et al., 1999). A large difference of microbiota in fecal contents of the dugong and cattle was also observed for the compositional percentage of *Clostridium* rRNA Cluster IV (12% and 46%), while that of *Cytophaga-Flexibacter-Bacteroides* was similar for both animals (Ozutsumi et al., 2002).

In contrast, the similar compositional percentages of *Firmicutes*, *Cytophaga-Flexibacter-Bacteroides*, and *Clostridium* rRNA Subcluster XIVa were observed for the dugong and horse, hind gut fermenters, for which the latter showed values of 72%, 20%, and 37%, respectively (Daly et al., 2001). There was a similar observation of the mirobiota in human vegetarian feces, which showed 90.7% of *Fermicutes*, 59.6% of *Clostridium* rRNA Subcluster XIVa, and 6.0% of *Cytophaga-Flexibacter-Bacteroides* (Hayashi et al., 2002b). However, no bacterial species contributing to the fiber degradation in the proximal colon was identified in this study, suggesting that the fiber-degrading bacteria, such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes*, may be a minor species, at least less than detection levels. One hundred and eighty-six of the clones were found to belong to Subcluster XIVa, which includes large numbers of butyrate-producing bacteria and *Ruminococcus* sp. Since the higher proportion of butyric acid in the intestinal contents and cultures of feces of dugongs as compared to that of other animals was previously observed (Goto et al., 2004b; Murray et al., 1977), some of the bacteria in the Subcluster XIVa may be associated with decomposition of the fiber and butyric acid generation. Some clones in Cluster IV were comparatively closer to *Ruminococcus flavefaciens*, although there was no evidence of similarity ($>$97%) with the known fiber-degrading bacteria. In previous study, $10^9$–$10^{10}$ colony forming units $g^{-1}$ of cellulose-utilizing bacteria were detected under a culture method (Goto et al., 2004b). The Cluster IV and XIVa population might account for cellulose-utilizing bacteria population.

Within the same group of hindgut fermenters including the horse, manatee, and dugong, the bacterial flora may be varied according to several complicated factors. The major ones are species, age, habitat, eating habit, digestive tract, and tract position. The large capacity of cecum, proximal colon, and distal colon in the dugong (Kamiya et al., 1979) can therefore induce dispersive bacterial biota of the individual, consistent with the complexity of the establishment of the microbiota in the digestive tract which was indicated for human feces (Hayashi et al., 2002a; Kageyama and Benno, 2000) and pigs (Simpson et al., 2000). Simpson et al. (2000) reported distinct differences of the bacterial biota in feces of the pigs produced from the same parent and fed the same diets. Thus, detailed comparisons of the compositional percentage of the microbiota in the digestive tract of different animal species are needed not only in the literature but also in experiments of their nutrition, digestion, and metabolisms.

Large differences of the bacterial biota were also observed between samples collected in May 2000 and October 2004 and/or August 2005. In May 2000, *Mollicutes* (34–37%), nearest to *Bulleidia moorei* AHP13983 (Downes et al., 2000; Kageyama and Benno, 2000), was decreased in 2004 or 2005. It was not clear whether it was due to seasonal variations, which can be associated with seasonal variations in chemical compositions and digestion potential of eelgrass fed, or changes of the physiological and/or digestion metabolism of the dugong, which can be associated with aging of the animal. There was, however, no report that the feeding and holding conditions at Toba Aquarium greatly changed in last several years or that, except for immature animals, the bacterial biota in the digestive tracts of grass-eating animals greatly changes with aging. As indicated by severe polarizations of the bacterial biota of the captive dugong, in which one phylotype occupied over 30% of the total in the samples of this study, a sole feeding of eelgrass at the aquarium would be easily reflected in the microbiota of the dugong. Thus, greater quantitative and qualitative variations of the microbiota in the hindgut between captured and wild-living dugongs should be certainly expected.

In this study, the whole biota of the dugong feces was characterized by 16S rDNA library sequence, indicating the presence and new findings of unknown species in the hindgut of the dugong. Technical procedures for bacterial DNA extraction used in this study were also relevant to overcome differences of cell wall

structure such as gram-positive and gram-negative bacteria in the fecal samples of a dugong. Further research is needed to isolate and elucidate the function and activity of each colony, especially in relation to fiber digestion of this marine herbivore.

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