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Isolation and identification of *Bifidobacterium* species from feces of captive chimpanzees

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Recently, gut-dwelling bifidobacteria from chimpanzees, which are phylogenetically close to humans and have feeding habits similar to humans, have been frequently investigated. Given this, we speculated that like humans, chimpanzees would have a unique diversity of bifidobacteria. We herein describe a taxonomically novel member of bifidobacteria isolated from fecal samples of captive chimpanzees. Bifidobacteria were detected in all fecal samples by quantitative polymerase chain reaction. A *Bifidobacterium pseudolongum*-like species, which could not be detected using *B. pseudolongum*-specific primers targeting the *groEL* gene sequence, was dominant in the feces of five chimpanzees. Seven bifidobacterial strains were isolated from this group of five chimpanzees, and all isolates were identified as *B. pseudolongum*. *B. pseudolongum* has previously often been isolated from non-primate animals as well as humans; however, here we demonstrate its presence in a nonhuman primate species.

Key words: *Bifidobacterium pseudolongum*, chimpanzee feces, 16S rRNA gene sequence, clone library, *groEL* gene sequence

INTRODUCTION

Bifidobacteria have been isolated from sewers [1], probiotic products [2], anaerobic digesters [3], etc., but the intestine of mammals, including humans, is considered to be their common habitat. Currently, over 40 *Bifidobacterium* species or subspecies are recognized [4]. In the human gut microbiota, common *Bifidobacterium* species include *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum*, *B. pseudocatenulatum*, *B. pseudolongum*, and *B. thermophilum* [4, 5]. Strains of these species are vaginally transmitted from mothers to infants at birth, and human milk contains oligosaccharides that can be specifically used by bifidobacteria (i.e., *B. bifidum*, *B. breve*, and *B. longum*) to become dominant members of the gut microbiota in breast-fed infants [6, 7]. Such evidence implies that bifidobacteria play a very important role in human health and longevity [8].

Recently, bifidobacteria from the guts of chimpanzees, which are closely related to humans and have feeding habits similar to humans, have been frequently investigated. To date, only four known *Bifidobacterium* species (i.e., *B. angulatum, B. catenulatum, B. pseudocatenulatum*, and *B. dentium*) have been identified in the guts of chimpanzees [9–11]; we speculated that like humans, chimpanzees would have a unique diversity of bifidobacteria. We herein describe the successful isolation of bifidobacteria from the feces of captive chimpanzees and clarify the taxonomic positions of these isolates.

MATERIALS AND METHODS

Subject animals

We collected fecal samples from captive chimpanzees captive at the Adventure World, Shirahama, Japan. The chimpansees included four adult females (C, H, T, and Y) and 2 adult males (J and K), and their ages ranged between 10 and 40 years.

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Table 1. Reference and type strains used in this study

Strain	Origin
Bifidobacterium pseudolongum subsp. *pseudolongum* JCM 1205^T	Rat feces
B. pseudolongum subsp. *pseudolongum* JCM 1264	Mouse feces
B. pseudolongum subsp. *pseudolongum* JCM 1266	Chicken feces
B. pseudolongum subsp. *globosum* JCM 5820^T	Bovine rumen
B. pseudolongum subsp. *globosum* JCM 7089	Bovine rumen
B. pseudolongum subsp. *globosum* JCM 7092	Bovine rumen

Fecal collection and sample preparation

Fecal samples were collected in March 2015 and June 2015 and were generously provided both times by the breeding staff of Adventure World, Shirahama, Wakayama, Japan. For the first lot, a portion of fresh feces (5–10 g) from each chimpanzee was scooped into an appropriately labeled plastic bag immediately after defecation and kept at −20°C before DNA extraction for subsequent molecular analyses. For the second lot, fresh feces (approximately 100 mg) from each chimpanzee were collected using commercial swabs and transport medium (BBL CultureSwab Plus, Becton Dickinson Italia, Milan, Italy), and the swabs were sent to our laboratory within 2 days before being subjected to isolation of viable bifidobacteria.

Reference bacterial strains

A total of six strains, including type or reference strains of the two subspecies of *B. pseudolongum* (i.e., *pseudolongum* and *globosum*), were used to help clarify the taxonomic position of the strains isolated from the chimpanzees (Table 1). Moreover, another 11 strains of 11 *Bifidobacterium* species were used as standards for quantitative PCR (qPCR) analysis (Table 2).

DNA preparation

Prior to DNA isolation, samples were mixed with 9 volumes of PBS. Whole genomic DNA from each fecal sample or bacterial culture was prepared following the method reported by Marmur *et al.* [12]. In brief, a 200-µl aliquot of each fecal solution or bacterial culture was transferred to sterile bead-beating tubes containing 300 mg of glass beads (0.1 mm in diameter). This was added to approximately 500 µl of TE-saturated phenol, 250 µl of lysis buffer, and 50 µl of 10% sodium dodecyl sulfate. After centrifugation at 10,000 g for 5 min, the upper layer was transferred to a new tube, and 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was then added to the tube and centrifuged at 10,000 g for 5 min. The upper aqueous layer was carefully collected in a new tube. The samples were shaken in a FastPrep-24 Instrument (MP Biomedicals SARL, Illkirch, France) for 30 sec at maximum speed. DNA was precipitated by adding 275 µl of isopropyl alcohol and a 1/10 volume of 3 M sodium acetate into the tube at −20°C for 10–15 min. The pellet was washed with 70% ice-cold ethanol by centrifugation at 10,000 g for 5 min, and DNA was dried under vacuum. DNA was subsequently dissolved again in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

qPCR analysis

qPCR was performed using a Thermal Cycler Dice Real Time System (Takara Bio Inc., Ohtsu, Japan). Primer pairs targeting a part of the 16S rRNA gene or the *groEL* gene for 12 bifidobacterial species were designed as reported by Junick *et al.* [4] and Matsuki *et al.* [13] (Table 2). For each assay, 1 µl of DNA solution was added to 9 µl of a PCR mixture containing 5 µl of THUNDERBIRD^{TM} SYBR® qPCR Mix (Toyobo, Osaka, Japan), 3.6 µl of distilled water, and 200 nM of each primer. The PCR conditions for the 12 bifidobacterial species are listed in Table 3. The total number of *Bifidobacterium* species was quantified using the 16S rRNA gene-targeted primer pair g-Bifid-F/g-Bifid-R designed as reported by Matsuki *et al.* [13] (Table 2).

Analysis of 16S rRNA gene clone libraries

The 16S rRNA gene fragments of the *Bifidobacterium* species fragments in fecal samples were amplified using a conventional PCR method with the primer pair g-Bifid-F/g-Bifid-R (Table 2). PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) and cloned to the TA cloning site of the pGEM-T Easy Vector (Promega Benelux, Leiden, Netherlands). Resulting plasmids were introduced into *Escherichia coli* DH5α cells (Takara Bio Inc., Ohtsu, Japan) via heat shock. The recombinant *E. coli* strain was grown at 37°C in Luria-Bertani (LB) broth (Becton Dickinson and Company, Sparks, MD, USA) for 1 hr. Cultures were spread on LB agar plates containing 100 µg/ml ampicillin (Wako Pure Chemical Industries, Osaka, Japan) and 400 pM 5-bromo-4-chloro-3- indolyl-

Table 2. Sequences of primers used in qPCR analysis

Target bacterial group	Sequence	Target gene	Size (bp)	Bacterial strains using as standard	Reference
Genus *Bifidobacterium*	g-Bifid-F: 5'-CTCCTGGAAACGGGTGG-3' g-Bifid-R: 5'-GGTGTTCTTCCCGATATCTACA-3'	16S rRNA gene	549–563	*Bifidobacterium catenulatum* JCM 1194^T	[13]
Bifidobacterium adolescentis	F1: 5'-CTCCAGTTGGATGCATGTC-3' F2: 5'-TCCAGTTGACCGCATGGT-3' R: 5'-CGAAGGCTTGCTCCCAGT-3'	16S rRNA gene	279	*Bifidobacterium adolescentis* JCM 1275^T	[13]
Bifidobacterium angulatum	F: 5'-CAGTCCATCGCATGGTGGT-3' R: 5'-GAAGGCTTGCTCCCCAAC-3'	16S rRNA gene	275	*Bifidobacterium angulatum* JCM 7096^T	[13]
Bifidobacterium bifidum	F: 5'-CCACATGATCGCATGTGATTG-3' R: 5'-CCGAAGGCTTGCTCCCAAA-3'	16S rRNA gene	278	*Bifidobacterium bifidum* JCM 1255^T	[13]
Bifidobacterium breve	F: 5'-CCGGATGCTCCATCACAC-3' R: 5'-ACAAAGTGCCTTGCTCCCT-3'	16S rRNA gene	288	*Bifidobacterium breve* JCM 1192^T	[13]
Bifidobacterium dentium	F: 5'-ATCCCGGGGGTTCGCCT-3' R: 5'-GAAGGGCTTGCTCCCGA-3'	16S rRNA gene	387	*Bifidobacterium dentium* JCM 1195^T	[13]
Bifidobacterium animalis	F: 5'-CACCAATGCGGAAGACCAG-3' R: 5'-GTTGTTGAGAATCAGCGTGG-3'	*groEL*	184	*Bifidobacterium animalis* JCM 1190^T	[4]
Bifidobacterium catenulatum	F: 5'-GGCTATCGTCAAGGAGCTCA-3' R: 5'-AGTCCAGATCCAAACCGAAAC-3'	*groEL*	188	*Bifidobacterium catenulatum* JCM 1194^T	[4]
Bifidobacterium gallicum	F: 5'-AGCTCGTCAAGTCCGCCAAGC-3' R: 5'-CATACCTTCGGTGAACTCGAGG-3'	*groEL*	188	*Bifidobacterium gallicum* JCM 8224^T	[4]
Bifidobacterium longum	F: 5'-CGGCGTYGTGACCGTTGAAGAC-3' R: 5'-TGYTTCGCCRTCGACGTCCTCA-3'	*groEL*	259	*Bifidobacterium longum* JCM 1217^T	[4]
Bifidobacterium pseudocatenulatum	F: 5'-AGCCATCGTCAAGGAGCTTATCGCAG-3' R: 5'-CACGACGTCCTGCTGAGAGCTCAC-3'	*groEL*	325	*Bifidobacterium pseudocatenulatum* JCM 1200^T	[4]
Bifidobacterium pseudolongum	F: 5'-CRATYGTCAAGGAACTYGTGGCCT-3' R: 5'-GCTGCGAMGAKACCTTGCCGCT-3'	*groEL*	312	*Bifidobacterium pseudolongum* JCM 1205^T	[4]
Bifidobacterium thermophilum	F: 5'-ACTGGTCGCTTCCGCCAAGGATG-3' R: 5'-CCARGTCAGCMAGGTGRACGATG-3'	*groEL*	326	*Bifidobacterium thermophilum* JCM 1207^T	[4]

Table 3. PCR conditions of qPCR analysis

Target bacterial group	PCR conditions
Bifidobacterium	94°C, 5 min; 40 cycles (94°C, 20 sec, 65°C, 20 sec; 72°C, 50 sec); 72°C, 10 min
Bifidobacterium adolescentis	94°C, 5 min; 40 cycles (94°C, 20 sec, 65°C, 20 sec; 72°C, 50 sec); 72°C, 10 min
Bifidobacterium angulatum	94°C, 5 min; 40 cycles (94°C, 20 sec, 65°C, 20 sec; 72°C, 50 sec); 72°C, 10 min
Bifidobacterium bifidum	94°C, 5 min; 40 cycles (94°C, 20 sec, 65°C, 20 sec; 72°C, 50 sec); 72°C, 10 min
Bifidobacterium breve	94°C, 5 min; 40 cycles (94°C, 20 sec, 65°C, 20 sec; 72°C, 50 sec); 72°C, 10 min
Bifidobacterium dentium	94°C, 5 min; 40 cycles (94°C, 20 sec, 65°C, 20 sec; 72°C, 50 sec); 72°C, 10 min
Bifidobacterium animalis	94°C, 3 min; 30 cycles (94°C, 30 sec, 65°C, 30 sec; 72°C, 30 sec); 72°C, 10 min
Bifidobacterium catenulatum	94°C, 3 min; 30 cycles (94°C, 30 sec, 47°C, 30 sec, 72°C, 30 sec); 72°C, 10 min
Bifidobacterium gallicum	94°C, 3 min; 30 cycles (94°C, 30 sec, 57°C, 30 sec, 72°C, 30 sec); 72°C, 10 min
Bifidobacterium longum	94°C, 3 min; 30 cycles (94°C, 30 sec; 66°C, 30 sec, 72°C, 30 sec); 72°C, 10 min
Bifidobacterium pseudocatenulatum	94°C, 3 min; 30 cycles (94°C, 50 sec, 64°C, 50 sec, 72°C, 50 sec); 72°C, 10 min
Bifidobacterium pseudolongum	94°C, 3 min; 30 cycles (94°C, 30 sec, 49°C, 30 sec, 72°C, 30 sec); 72°C, 10 min
Bifidobacterium thermophilum	94°C, 3 min; 30 cycles (94°C, 40 sec, 65°C, 40 sec, 72°C, 40 sec); 72°C, 10 min

β-D-galactopyranoside (Sigma-Aldrich, St, Louis, MO, USA) and grown overnight at 37°C. White colonies were subcultured to new LB agar plates using an autoclaved toothpick and then incubated at 37°C for 16 hr.

At least 10 colonies were selected from each library and then grown overnight in LB broth. Plasmids were harvested and purified from the overnight cultures using a plasmidPrep Mini Spin Kit (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). Sequencing of inserted fragments was performed with the vector-specific primers T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-CAAGCTATTTAGGTGACACTATAG-3′) using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, UK) and Applied Biosystems 3100xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

Isolation of tentatively bifidobacterial strains from chimpanzee feces

Fresh feces of six chimpanzees were serially diluted with 1×PBS and streaked on TOS propionate (Yakult, Tokyo, Japan) and BL (Nissui Pharmaceutical, Tokyo, Japan) agar plates with platinum loops. The agar plates were then anaerobically incubated (Mitsubishi Gas Chemical Company, Tokyo, Japan) at 37°C for 48 hr. Well isolated colonies that appeared to be bifidobacteria were tentatively identified as *Bifidobacterium* strains for further taxonomic analyses as described below.

PCR amplification and 16S rRNA sequencing of isolates

A large, continuous fragment (approximately 1440 bp) of the 16S rRNA gene from isolates was obtained using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR products were purified using a NucleoSpin Extract II Kit (Macherey-Nagel, Duren, Germany). Sequencing was performed with the primers 27F, 518F (5′-CCAGCAGCCGCGGTAATACG-3′), 1100R (5′-GGGTTGCGCTCGTTG-3′), and 1492R using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and Applied Biosystems 3100xl Genetic Analyzer (Applied Biosystems).

PCR amplification and sequencing of groEL

A large, continuous fragment (approximately 1600 bp) of the *groEL* gene from our isolates was obtained using the primers BpgroF (5′-TCATTGAATATGATGAGGAAGCA-3′) and BpgroR (5′-AACAGCCTCGGTCGTCAG-3′), which were designed for amplification of the *B. pseudolongum groEL* gene based on the complete genome sequence *B. pseudolongum* PV-8 (accession number CP007457.1) [15]. Amplification reactions were performed using the following program: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, with a final extension period at 72°C for 10 min. PCR products were then purified using a NucleoSpin Extract II Kit (Macherey-Nagel, Duren, Germany). Sequencing of the purified PCR products was performed with the same primers as used for PCR using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) and Applied Biosystems 3100xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

Table 4. Quantification of *Bifidobacteirum* species in feces of captive chimpanzees

Target gene	Target species	Individual chimpanzees*					
		H	C	J	Y	T	K
16S rRNA gene	Total *Bifidobacterium*	8.89	8.58	7.49	9.87	10.26	7.58
	B. adolescentis	−	−	−	−	−	−
	B. angulatum	−	−	−	−	−	−
	B. bifidum	−	−	−	−	−	−
	B. breve	−	−	−	−	−	−
	B. dentium	−	−	−	−	−	−
groEL	*B. animalis*	−	−	−	−	−	−
	B. catenulatum	−	−	−	−	−	−
	B. gallicum	−	−	−	−	−	−
	B. longum	−	−	−	−	−	−
	B. pseudocatenulatum	−	−	−	8.16	−	−
	B. pseudolongum	−	−	−	−	6.80	−
	B. thermophilum	−	−	−	−	−	−

*Values indicate the $\log_{10}$ cells/g in fecal samples.
A dash (−) indicates that the value was less than the detection limit.

Phylogenetic analysis

Obtained 16S rRNA and *groEL* sequences were aligned via ClustalW using the MEGA5 software package [16]. Sequences were subjected to similarity search analysis using the BLAST algorithm in the NCBI database. Phylogenetic trees based on *groEL* sequences were constructed using the neighbor-joining method [17] with MEGA5. Other phylogenetically related *Bifidobacterium groEL* sequences retrieved from GenBank were also included. The stability of the groupings was estimated via bootstrap analysis (1,000 replications).

Whole genome sequences of isolates

Whole genome sequences of 3 of our isolates (T-1, J-1, and Y-1) were determined using the Illumina/Solexa technology. An average of 0.75–3.38 million paired-end reads of 262.1 bp were generated by MiSeq (Illumina, San Diego, CA, USA). All generated reads were assembled into contigs using the CLC Genomics Workbench software v. 6.0 (CLC bio, Aarhus, Denmark).

Calculation of average nucleotide identity (ANI) values

The degree of pairwise genome-based relatedness was calculated as an ANI value following the BLAST-based ANI calculation method using the JSpecies software [18]. ANI values for *Bifidobacterium* strains, including those designated to *B. pseudolongum*, whose genome sequences were available in the GenBank database were calculated.

Biochemical characterization

Physiological and biochemical characteristics of isolates were determined with commercially available identification kits, API 20A and Rapid ID 32A (bioMérieux, Lyon, France), according to manufacturer's protocols.

Nucleotide sequence accession number

The determined sequences of the *groEL* gene and 16S rRNA gene of the isolates from the captive chimpanzees were completely identical. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *groEL* gene sequences of strain T-1 are LC210630 and LC210631, respectively. The GenBank/EMBL/DDBJ BioProject ID for the draft genome sequences of strains T-1, J-1, and Y-1 is PRJDB5473.

RESULTS

Quantification of Bifidobacterium species in chimpanzee fecal samples

The number of *Bifidobacterium* species in 1 g chimpanzee feces was quantified via qPCR, and the detection limit was taken as 1×10^5 cells/g. Although more than 3.1×10^7 cells/g of *Bifidobacterium* was detected in all fecal samples, *B. pseudocatenulatum* and *B. pseudolongum* were detected in the feces of chimpanzee Y (1.4×10^8 cells/g for the *groEL* sequence) and chimpanzee T (1.5×10^6 cells/g for the *groEL* sequence), respectively (Table 4). No other species were detected from the fecal samples. Although a certain number of *Bifidobacterium* species were detected all together in genus-specific qPCR, species-specific qPCR could not detect as many bifidobacterial species (Table 4).

Analysis of 16S rRNA gene clone libraries

To investigate a cause for the numerical difference between the total number of *Bifidobacterium* and bifidobacterial species in chimpanzee fecal samples, we performed a 16S rRNA gene clone library analysis. More than 60 clones were sequenced. The obtained sequences were subjected to a similarity search analysis using the BLAST algorithm in the NCBI database. Comparative 16S rRNA gene sequence analysis revealed 99–100% sequence similarities between all clones and *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T, and the clones and *B. pseudomlongum* subsp. *pseudolongum* JCM 1205^T formed a cluster independently from other closely related species.

Isolation and identification of bifidobacterial strains from chimpanzee feces

A total of seven bifidobacterial strains (H-1, H-2, T-1, T-2, J-1, C-1, and Y-1) were obtained from five of the chimpanzees (C, H, J, T, and Y). Approximately 1350 bp of the 16S rRNA gene sequence of each isolate was determined and then compared with published sequences obtained from GenBank nucleotide databases using the BLAST algorithm. Because our phylogenetic analysis based on 16S rRNA gene sequences showed that all the strains possessed high sequence similarities (99.5%) to *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T, the isolates were tentatively identified as *B. pseudolongum*.

GroEL sequence analysis

The 16S rRNA gene has been widely used as a valuable tool for bacterial identification [13]. However, the resolution power of the 16S rRNA gene among closely related species is limited. Some *Bifidobacterium* species reveal a relatively high 16S rRNA gene sequence identity [13]. The *groEL* gene has been previously used to differentiate *Bifidobacterium* species [4, 13]. Thus, we designed primers targeting the *groEL* gene of *B. pseudolongum* based on the complete genome sequence of *B. pseudolongum* PV-8. Approximately 1,450 bp of the *groEL* gene of each isolate was determined, and all the sequences were completely identical.

Comparative sequence analysis revealed 95.6–98.6% sequence similarities for *groEL* between our isolates and known *B. pseudolongum* strains, including *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T (98.6% of sequence identity) and *B. pseudolongum* subsp. *globosum* JCM 5820^T (95.6% of sequence identity). Such results demonstrated their high genetic relatedness, but we also observed marked differences from those of other *Bifidobacterium* species, i.e., less than 87.7% sequence similarities. Moreover, these isolates and *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T formed one cluster among *Streptococcus* species in phylogenetic trees inferred from *groEL* sequence comparisons (Fig. 1).

Whole genome sequence analysis

To further elucidate the taxonomic position of isolates obtained from chimpanzee feces, draft genome sequences were generated. Whole genome sequences of strains T-1, J-1, and Y-1 were determined using Illumina MiSeq technology. An average of 0.75–3.38 million paired-end reads, with a length of 262.1 bp, were generated via the MiSeq system. The resulting draft genomes of the three isolates had 11 or 12 contigs with 200−1,000-fold coverage, and the genome size ranged from 1.94 to 1.95 Mb. The degree of pairwise genome-based relatedness was calculated as the ANI value according to the BLAST-based ANI calculation method. The ANI values among isolates, T-1, J-1, and Y-1 and the strain *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T ranged from 98.6% to 99.9% (Table 5). These ANI values were higher than the 95% ANI cut-off value for bacterial species proposed by Goris *et al.* [19]. On the other hand, the ANI values between our isolates and *B. pseudolongum* subsp. *globosum* JCM 5820^T were closer (95.5%) to the proposed ANI cut-off value for bacterial species (Table 5).

Biochemical characterization

The differential biochemical characteristics among the strains used in this study are shown in Table 6. Based on analyses with the API 20A and Rapid ID 32A systems, our isolates, JCM 1264, JCM 7089, and JCM 7092, possessed similar carbohydrate fermentation patterns, with the exception of the strain C-1. Strain C-1 possessed similar carbohydrate fermentation patterns similar to those of *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T [20]. Combining *groEL* phylogeny, genome sequence comparative analysis, and carbohydrate fermentation patterns, we concluded that our J1, T1 and Y1 isolates were *B. pseudolongum* subsp. *pseudolongum* and that the other strains were also identical or closely related to this subspecies.

DISCUSSION

Bifidobacteria are known to establish a balance in the gut microbiota and confer health benefits to the host. They represent one of the largest bacterial groups within Actinobacteria, and most *Bifidobacterium*

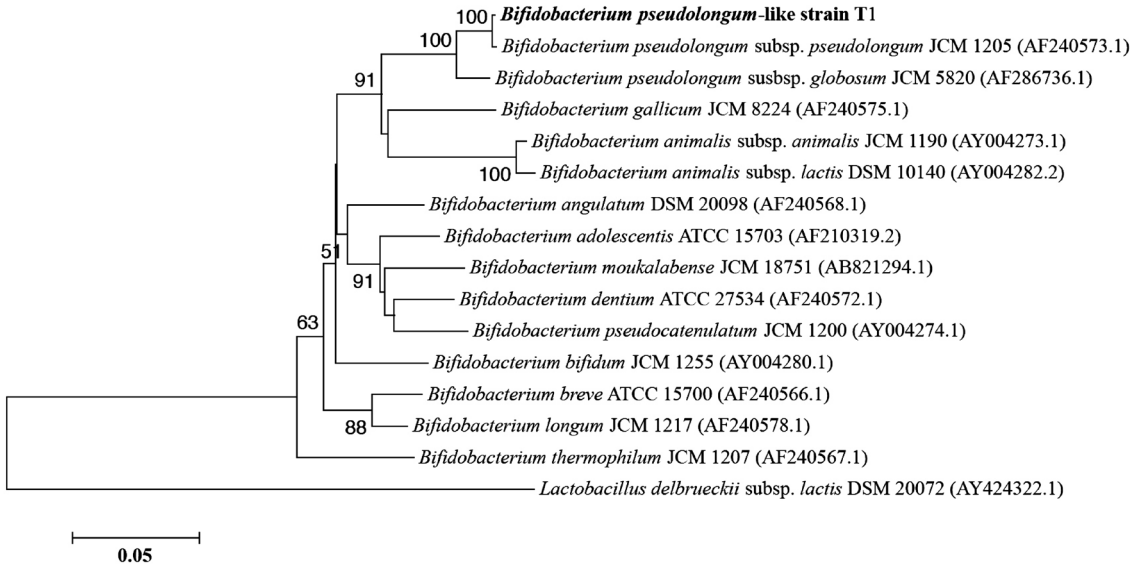

Fig. 1. The *groEL* gene sequence-based phylogenetic tree of *Bifidobacterium pseudolongum*-like strain T1 and selected type strains of species of the genus *Bifidobacterium*.

The neighbor-joining tree is shown here with bootstrap support values; only values >50% are shown. Bar, 5% sequence divergence. Accession numbers of the reference sequences used in the phylogenetic analysis are shown in parentheses. *Lactobacillus delbrueckii* is shown as an outgroup.

Table 5. Average nucleotide identity (ANI) values among the genome sequences of genus *Bifidobacteirum*

Strain names	PV8-2	J-1	T-1	Y-1	JCM 1205^T	JCM 5820^T	JCM 1190^T
B. pseudolongum PV8-2 (CP007457)	---	93.82	93.81	93.82	93.45	95.79	86.01
J-1		---	**99.9**	**99.91**	**98.62**	95.52	86.93
T-1			**---**	**99.94**	**98.61**	95.52	86.87
Y-1				**---**	**98.63**	95.53	86.89
B. pseudolongum subsp. *pseudolongum* JCM1205^T (NZ_JGZH01000001-11)					---	94.75	86.13
B. pseudolongum subsp. *globsum* JCM5820^T (NZ_JGZG01000001-26)						---	87.04
B. animalis JCM1190^T (CP002567)							---

*Bold: >98%, The DDBJ/EMBL/GenBank accession numbers of reference and type strains are given in parenthese.

species are found in the gastrointestinal tract of humans and animals and in the hindgut of insects. However, little information is available regarding the intestinal microbiota of chimpanzees. Recently, limited sequence analyses and isolation experiments suggested the presence of *B. catenulatum*-, *B. pseudocatenulatum*-, and *B. angulatum*-like species in wild chimpanzees from Bossou, Guinea [9, 10]. In this study, we successfully isolated the *B. pseudolongum* subsp. *pseudolongum* strains from the feces of chimpanzees living in captivity. *B. pseudolongum* comprises two subspecies, *pseudolongum* and *globosum*, and is often detected in the feces of various animals, such as rats, pigs, sheep, cows [20], dogs, and cheetahs [11]. With respect to primates, *B. pseudolongum* has only been isolated from humans [4, 14]. To the best of our knowledge, this is the first report of *B. pseudolongum* being identified in a nonhuman primate species. Thus, our findings indicated the possibility of chimpanzees possessing a unique diversity of bifidobacteria. Via 16S rRNA gene clone library analysis, all analyzed clones were shown to have possessed 16S rRNA fragments highly identical to *B. pseudolongum*. Thus, *B. pseudolongum* may be predominant in the intestinal bifidobacterial flora of these chimpanzees.

In this study, we also assessed the *groEL* gene. This housekeeping gene undergoes mutation more easily than 16S rRNA, which may help in differentiating

Table 6. Characteristics that differentiate among the strains used in this study

	1	2	3	4	5	6	7	8	9	10	11	12	13
Hydrolysis of arginine	−	−	−	−	−	+	+	−	+	−	+	−	+
Hydrolysis of esculin	−	+	−	+	+	+	+	+	+	+	+	−	+
Production of (ID 32A) of													
β-Glucosidase	nt	+	+	−	+	−	−	−	+	−	+	−	+
β-Galactosidase	nt	+	+	+	+	+	−	+	+	+	+	+	+
α-Glucosidase	nt	−	+	+	+	+	−	−	−	+	−	+	−
α-Galactosidase	nt	+	+	+	−	−	−	+	−	+	−	+	−
α-Arabinosidase	nt	−	+	−	−	−	−	+	−	+	−	+	−
Proline arylamidase	nt	+	+	+	+	+	+	+	−	+	−	+	−
Pyroglutamic acid arylamidase	nt	−	−	−	+	+	+	−	+	−	+	−	+
Argininec arylamidase	nt	+	+	+	+	+	+	−	+	−	+	+	+
Alanine arylamidase	nt	−	+	−	+	+	+	−	+	+	+	−	−
Production (API 20A) of acid from													
Salicin	+	+	+	+	+	+	+	+	+	+	+	−	+
Mannitol	−	+	−	+	+	+	+	+	+	+	+	−	+
Xylose	+	−	−	+	−	−	−	+	−	−	+	+	−
Cellobiose	−	+	−	+	+	+	+	+	+	+	+	−	+
Raffinose	+	−	+	+	−	−	−	+	−	−	−	+	−
Mannose	−	+	+	+	+	+	+	+	+	+	+	−	+
Trehalose	−	+	−	+	+	+	+	+	+	+	+	−	+

Strains: 1, *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T; 2, *B. pseudolongum* subsp. *pseudolongum* JCM 1264; 3, *B. pseudolongum* subsp. *pseudolongum* JCM 1266; 4, *B. pseudolongum* subsp. *globsum* JCM 5820^T; 5, *B. pseudolongum* subsp. *globsum* JCM 7089; 6, *B. pseudolongum* subsp. *globsum* JCM 7092; 7, H-1; 8, H-2; 9, T-1; 10, T-2; 11, J-1; 12, C-1; 13, Y-1.
Phenotypic data for strains 2 to 13 are from this study. Data for strain 1 are from Yaeshima *et al.* [20]. +, positive reaction; –, negative reaction; nt, not tested.

between species [15]. Via our qPCR analysis of *groEL*, *B. pseudolongum* was not detected in the feces of the captive chimpanzees, although *B. pseudolongum* was the predominant species detected according to our clone library analysis. The sequence analysis of the *groEL* gene revealed that there is a nucleotide mutational point near the 3′-end of the *B. pseudolongum*-specific primers used in this study. This mutation site might have inhibited the qPCR extension step in our analysis. We presumed that this is the reason why *B. pseudolongum* was not detected via qPCR using our primers targeting *groEL*. Thus, we designed primers that could specifically amplify the partial sequence of the 16S rRNA gene of *B. pseudolongum* and *B. animalis* and re-performed the qPCR analysis. The bifidobacterial cell numbers based on this qPCR analysis were comparable to the total cell numbers of *Bifidobacterium* species in five samples (data not shown). At the moment, the distribution and role of *B. pseudolongum* in captive chimpanzees is unclear, although bifidobacteria are believed to be a common member of the chimpanzee intestinal microbiota [10]. Further study is required to clarify the unique diversity of bifidobacterial flora in chimpanzees.

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