Characteristics of intestinal microbiota of western lowland gorillas (*Gorilla gorilla gorilla*)

Ph. D. Dissertation

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1) *Bifidobacterium moukalabense* sp. nov. isolated from the faeces of wild western lowland gorilla (*Gorilla gorilla gorilla*) in Gabon (2014). Tsuchida, S., Takahashi, S., Nguema, PP., Fujita, S., Kitahara, M., Yamagima, J., Ngomanda, A., Ohkuma, M. and Ushida, K. *Int J Syst Evol Microbiol.* 64: 449-455.

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2) *Lactobacillus gorillae* sp. nov. isolated from the faeces of captive and wild western lowland gorillas (*Gorilla gorilla gorilla*) (2014). Tsuchida, S., Kitahara, M., Nguema, PP., Norimitsu, S., Fujita, S., Yamagiwa, J., Ngomanda, A., Ohkuma, M. and Ushida, K. *Int J Syst Evol Microbiol.* In Press: doi:10.1099/ijs.0.068429-0.

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3) Characterization of intestinal bacterial communities of western lowland gorillas (*Gorilla gorilla gorilla*), central chimpanzees (*Pan troglodytes troglodytes*) and a forest elephant (*Loxodonta africana cyclotis*) living in Moukalaba-Doudou National Park in Gabon (2014). Tsuchida, S. and Ushida, K. *Tropics*. In Press.

Chapter 1

General introduction

1-1 Intestinal microbiota in mammals

Intestinal microbiota of mammals develop complex ecosystems with vast diversity after birth that show large differences between animal species and individuals (Hooper 2004, Qin *et al*. 2010). In fact, 1,000 species (or, more precisely, operational taxonomic units) were detected in the human intestine (Rajilic-Stojanovic *et al*. 2007), and 100 trillion microorganisms reside in a human intestine, a number ten times greater than the total number of human cells in the body. These vast microbiota mutually interact with host human cells to promote many physiological responses of the host. Accordingly, humans are now recognized as being made up of superorganisms together with indigenous microbes (Hattori and Taylor 2009).

According to Hattori and Taylor, the phylum Bacteroidetes, such as *Bacteroides ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *B. caccae*, and *B. fragilis*, and the phylum Firmicutes, such as *Ruminococcus gnavus*, *R. torques*, and *Eubacterium ventriosum*, are the most active bacteria in adult human intestinal microbiota. In the infant, the phylum Firmicutes, such as *Clostridium ramosum*, *C. clostridioforme*, *R. gnavus*, *Lactobacillus johnsonii*, *L. acidophilus*, *Streptococcus agalactiae*, *S. pneumonia*, and *Enterococcus faecalis*, and the phylum Actinobacteria, such as *Bifidobacterium longum*, *B. catenulatum*, and *Collinsella aerofaciens*, are the most active bacteria in intestinal microbiota. These bacteria are mostly defined as commensal (*i.e.*, non-harmful) bacteria.

Intestinal microbiota, particularly the above-mentioned commensals, have

attracted the attention of many researchers, even those without a background in classical bacteriology, since the introduction of the culture-independent approach and metagenomic analyses realized by Next Generation Sequencing. This is because intestinal microbiota play an important role in digestion, absorption of nutrients, and maintaining the health of the host. Indeed, many studies are concerned with gut microbiota in order to characterize their comprehensive relationship to metabolic disorders and chronic diseases in view of the loss of health-promoting indigenous bacteria (Ley *et al*. 2005, Ley *et al*. 2006, Turnbaugh *et al*. 2008, Kellermayer 2013, Andoh *et al*. 2007, Wen *et al*. 2008). In most of these studies, the role of commensal bacteria in the development of gut-associated lymphoid tissue (GALT) has been focused on explaining their health promotion (Rhee *et al*. 2004). In addition to human and human models such as rats or mice, intestinal microbiota have been studied intensively in livestock, including some pet animals, due to their economic importance (Zhou *et al*. 2007, Dowd *et al*. 2008, Turnbaugh *et al*. 2008, Hill *et al*. 2010, Handl *et al*. 2011).

Such an ecosystem may evolve in a host-specific manner, as shown in the earlier study by Mitsuoka and Kaneuchi (1977). Host-specific development of intestinal microbiota is most likely caused by the host's digestive system (Stevens and Hume 1995) together with membrane glycoprotein of epithelial cells (Aissi *et al*. 2001, Laparra and Sanz 2009) and food habits (Ley *et al*. 2008).

1-2 Lactic acid bacteria in mammals

Lactic acid bacteria, namely bifidobacteria and lactobacilli, are common members of the gastrointestinal microbiota in a wide range of mammals (Mitsuoka and Kaneuchi 1977), and each animal species hosts different species of lactic acid bacteria

in its gastrointestinal tract (Endo *et al*. 2010).

Many studies are concerned with the host-beneficial functions of lactic acid bacteria; for example, the immune-stimulating effect caused by the interaction between lactic acid bacteria and the intestinal mucosa of the host elevates the host's defense against pathogenic penetration (Yamamoto *et al*. 1996, Kelly *et al*. 2005, Uchida *et al*. 2006, Fink *et al*. 2007, Riboulet-Bisson *et al*. 2012).

As mentioned above, the application of studies on the intestinal microbiota has been limited to humans (and its model animals) and livestock, due to their economic importance. Information regarding lactic acid bacteria in other animals is scarce. As shown above, lactic acid bacteria are the most active component of commensal intestinal bacteria in human infants. However, it is still unknown why lactic acid bacteria were selected to play such an important role in host defense, why they have a particular ecological niche in the gastro-intestinal tract, etc. Their importance should be recognized with the notion of the co-evolution of lactic acid bacteria with their mammalian hosts. In this context, studying the lactic acid bacteria of other mammals, particularly our phylogenetic neighbor the great apes, is important.

As pioneers of intestinal microbiology, Mitsuoka and Kaneuchi (1977) isolated intestinal bacteria from various animal species using a fine-tuned culture-dependent method. They found that animals could be classified as either bifidobacterial- or lactobacillal-type animal. In the former, including humans, monkeys, guinea pigs, and chickens, bifidobacteria are the predominant lactic acid bacteria; in the latter, including pigs, mice, rats, hamsters, horses, and dogs, lactobacilli are predominant. Following their work, studies on the diversity of bifidobacteria and lactobacilli in herbivores, omnivores, and carnivores have been continued using culture-independent methods

(Lamendella *et al*. 2008, Walter 2008, Endo *et al*. 2010). In an intensive study by Lamendella *et al*., a range of domestic or captured animals such as humans, alpacas, llamas, beef cattle, dairy cattle, deer, goats, sheep, rabbits, hogs, horses, pigs, opossums, prairie dogs, bobcats, coyotes, domestic cats, domestic dogs, ferrets, Canadian geese, chickens, peacocks, pigeons, and turkeys was surveyed. *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, *B. infantis*, and *B. longum* were detected in not only humans but also a wide range of animals (Table 1-1). The distribution is interesting because very limited detection was observed in the ruminants. *B. adolescentis*, *B. catenulatum*, and *B. dentium* were the species detected in the ruminant. However, these three species were allochthonous species and all detected in humans and pigs. Interestingly, pigs were the host for many species of bifidobacteria, although pigs are categorized as lactobacillal-type animals. In their study, the level of bifidobacterial diversity in pigs was followed by that in humans. The reasons for this particular pattern of distribution are unknown.

L. acidophilus, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. salivarius*, *L. ruminis*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. plantarum*, *L. reuteri*, *L. fermentum*, *L. brevis*, *L. delbrueckii*, *L. sakei*, *L. vaginalis*, and *L. curvatus* are recognized as human-associated lactobacilli, although lactobacilli are not considered to be true inhabitants of the human GIT (Walter 2008). Nevertheless, lactobacilli are present in high numbers throughout the GIT of mice, rats, pigs, and chickens. Among the various lactobacilli, *L. reuteri* is considered to be an allochthonous lactobacillus (Walter 2008). In the study of the captured animals, lactobacilli were detected in all of the carnivores tested, but detection was limited to some omnivores and a few herbivores (Endo *et al*. 2010). *L. reuteri*, *L. johnsonii*, *L. salivarius*, *L. vaginalis*, and *L. ingluviei* were detected as dominant

lactobacilli in carnivores such as the cape fox, bat-eared fox, jackal, caracal, and lion. Interestingly, lactobacilli that are detected in plants and plant materials, such as *L. brevis*, *L. casei*, *L. parabuchneri*, *L. plantarum*, *L. sakei*, *Leuconostoc mesenteroides*, and *Leu. pseudomesenteroides*, were detected in omnivores such as raccoons, mongooses, marmosets, and tamarins.

Unfortunately, all of these studies were limited to livestock or captured wild animals, which caused a tremendous artificial effect on the development of intestinal microbiota. This artificial effect was clearly shown by comparisons between wild and captured chimpanzees (Uenishi *et al*. 2007). Such a difference was caused not only by the feeding procedure under conditions in captivity but perhaps also by the replacement of original bacteria with human-borne bacteria. *B. angulatum*-like bacteria were isolated from wild chimpanzees, while *B. dentium* was the major bifidobacteria detected in an infant chimpanzee artificially raised after birth (Uenishi *et al*. 2007, Ushida 2009).

1-3 Aim of this study

As discussed above, lactobacilli and bifidobacteria reside in a wide range of mammals as autochthonous intestinal bacteria. Therefore, some of the species in this group of bacteria should be regarded as allochthonous intestinal bacteria. In fact, the same species of lactic acid bacteria were detected in a wide range of animals (Lamendella *et al*. 2008, Walter 2008, Endo *et al*. 2010). However, it is still difficult to reveal how and what kind of selection was applied to the intestinal bacteria for the establishment of host-specific intestinal microbiota, although several prominent studies did reveal the distribution pattern of intestinal lactic acid bacteria. This limitation is probably caused by the selection of the host animals. In fact, studies were based only on

livestock and captured wild animals. Eating habits in native wild habitats are far from those in conditions of captivity. For example, wild plants have many protective chemicals to thwart predation (Bryant *et al*. 1992). Since these plants' secondary metabolites may affect the microbial community (Wallace 2004, Uhlik *et al*. 2013), consumption of vegetables and crops under conditions in captivity may allow the growth of bacteria sensitive to the plants' secondary metabolites. Moreover, the selection of target animals in the study did not account for phylogenetical ancestor-progeny relationships. Therefore, it is difficult to distinguish a vertically derived distribution (*i.e*., history of evolution: autochthonous) and horizontal transmission of a particular type of bacteria (allochthonous).

One of the aims of this study was to investigate lactic acid bacteria in non-human wild primates. We have conducted a study of lactic acid bacteria in wild chimpanzees prior to this study (Uenishi *et al*. 2007, Ushida 2009, Ushida *et al*. 2010). In the present study, the author intended to characterize the lactic acid bacteria in wild and captured western lowland gorillas. The identification and characterization of gorilla-specific (or common for gorillas and chimpanzees) lactic acid bacteria may further help us understand the particular relationship between humans and bifidobacterium. Humans harbor higher amounts of bifidobacteria in the GIT with a species-level diversity much larger than that of other animals. Characterization of gorilla-specific bifidobacteria at the level of the bacterial genome will reveal what kind of selection worked in the human GIT as it is compared with the genomes of human-associated bifidobacteria. Loss of genes or the creation of novel functionality will be shown by comparing the characteristics of gorilla-specific species and those associated with humans.

The common ancestor of gorillas and humans presented some 7–10 million years ago in the forest of Africa (Gaqneux *et al.* 1999, Stewart and Disotell 1998, Scally *et al.* 2012). Since then, gorillas have always stayed in the tropical rain forest, depending always on the fruits and leaves; however, our human ancestors left the forest for the savanna and a hunting-gathering subsistence (McHenry and Coffing 2000). The food habits of our ancestors seem to have shifted from frugivore/folivore to carnivore/omnivore. Our food habits further shifted from hunting-gathering to crop consumption after the creation of agriculture some 10,000 years ago (Eaton and Konner 1985). This evolution of food habits may have selected for particular bifidobacteria and/or induced the diversification of bifidobacteria. Therefore, the author intended to isolate bifidobacteria that are the original species in wild western lowland gorillas, because there have been no reports concerning bifidobacteria in wild gorillas thus far. If gorilla-specific bifidobacteria were obtained, the comparison between those bifidobacteria and those associated with humans would provide the answer to this question.

In addition to bifidobacteria, the author focused on lactobacilli in wild western lowland gorillas. As mentioned above, lactobacilli are not a predominant member of the human microbiota. It has yet to be determined whether gorillas are bifidobacterial- or lactobacillal-type animals. Accordingly, the author intended to study and isolate lactobacilli from wild and captive western lowland gorillas. If gorillas have a distinct type of lactobacilli, the same approach as for gorilla-specific bifidobacteria will help us understand the adaptation of intestinal bacteria through the evolution of human food habits.

The second aim of this study was to evaluate environmental effects on

intestinal microbiota. In this respect, the author intended to study the intestinal microbiota of western lowland gorillas, central chimpanzees, and a forest elephant in the wild by using pyrosequencing analysis because gorillas and chimpanzees in our study site are sympatric and depend on nearly the same food varieties. In general terms, the chemical components of the food seem to be similar for gorillas and chimpanzees. Elephants also depend mostly on similar foods in the forest. Therefore, the author included forest elephants in the study's out-group.

The study was conducted in Moukalaba-Doudou National Park (MDNP), Nyanga State, Gabon (Fig. 1-1). The park covers an area of 5,028 km^2. The study area covers about 120 km^2 in the southeastern part of the park at an altitude of 50–800 m. The research station was located at 2° 20′ and 10° 34′ E. The vegetation is a complex mosaic of semi-primary forest, secondary forest, *Musanga cecropioides*-dominated forest, savanna, and swamp (Iwata and Ando 2007). This area typically experiences two seasons: the rainy season from mid-October to May and the dry season from June to September. The mean annual rainfall (2002–2006) was 1,777 mm (range: 1,583–2,163 mm). The mean monthly minimum and maximum temperatures varied from 21.3°C to 24.1°C and 29.3°C to 33.7°C, respectively (Takenoshita *et al*. 2008).

In this study area, we observed a wide range of mammals: gorillas, chimpanzees, forest elephants, monkeys, mandrills, red river hogs, forest buffaloes, duikers, panthers, wild cats, and pangolins.

Table 1-1. Results from *Bifidobacterium* species-specific PCR assays using different animal fecal DNA extracts*

Animal type (n)[a]	No. of samples (%) detected with *Bifidobacterium* sp. - and group-specific primer[b]								
	B. adolescentis	*B. angulatum*	*B. bifidum*	*B. breve*	*B. catenulatum group*	*B. dentium*	*B. gallicum*	*B. infantis*	*B. longm*
Alpaca (2)	1 (50)	-	-	-	-	-	-	-	-
Beef cattle (14)	-	-	-	-	1 (7.1)	1 (7.1)	-	-	-
Canadian goose (20)	-	-	-	-	2 (10)	2 (10)	-	-	-
Chicken (29)	1 (3.4)	-	2 (6.9)	2 (6.9)	-	-	3 (10.3)	-	-
Coyote (11)	1 (9.1)	-	-	-	1 (9.1)	1 (9.1)	-	-	-
Dairy cattle (14)	4 (28.6)	-	-	1 (7.1)	2 (14.3)	8 (57.1)	1 (7.1)	-	-
Deer (17)	1 (5.9)	-	-	-	2 (11.8)	2 (11.8)	2 (11.8)	-	-
Domestic cat (10)	-	-	2 (20)	-	5 (50)	-	-	-	-
Domestic dog (15)	-	-	1 (6.7)	-	2 (13.3)	-	-	-	-
Goat (4)	-	-	-	-	2 (50)	2 (50)	-	-	-
Guinea pig (1)	-	-	-	-	-	1 (100)	-	-	-
Hog, feral (1)	-	-	-	-	-	1 (100)	-	-	-
Horse (16)	-	-	-	-	2 (12.5)	3 (18.8)	4 (25)	-	-
Human (19)	7 (36.8)	-	4 (21.1)	-	4 (21.1)	1 (5.3)	-	-	1 (5.3)
Pig (43)	13 (30.2)	11 (25.6)	22 (51.1)	2 (4.7)	20 (46.5)	15 (34.9)	7 (16.3)	2 (4.7)	2 (4.7)
Pigeon (4)	-	-	1 (25)	-	-	-	-	-	-
Possum (2)	-	-	-	-	-	1 (50)	-	-	-
Prairie dog (2)	-	-	-	-	-	1 (50)	-	-	-
Rabit (4)	-	1 (25)	2 (50)	1 (25)	1 (25)	1 (25)	1 (25)	-	-
Septic (9)	1 (11.1)	1 (11.1)	3 (33.3)	-	1 (11.1)	1 (11.1)	1 (11.1)	-	-
Sheep (8)	3 (37.5)	1 (12.5)	-	-	2 (25)	5 (62.5)	2 (25)	-	2 (25)
Squirrel (4)	-	-	-	-	-	1 (25)	-	-	-
Turkey (10)	-	1 (10)	-	-	-	-	-	-	-

* adapted and modified from Table 3 in Lamendella *et al*. 2008

[a] n, number of fecal samples tested for that animal type

[b] -, no amplification product was visualized for any of the samples from a given animal. The figures in parentheses are percentages of the amplification-positive products in the samples.

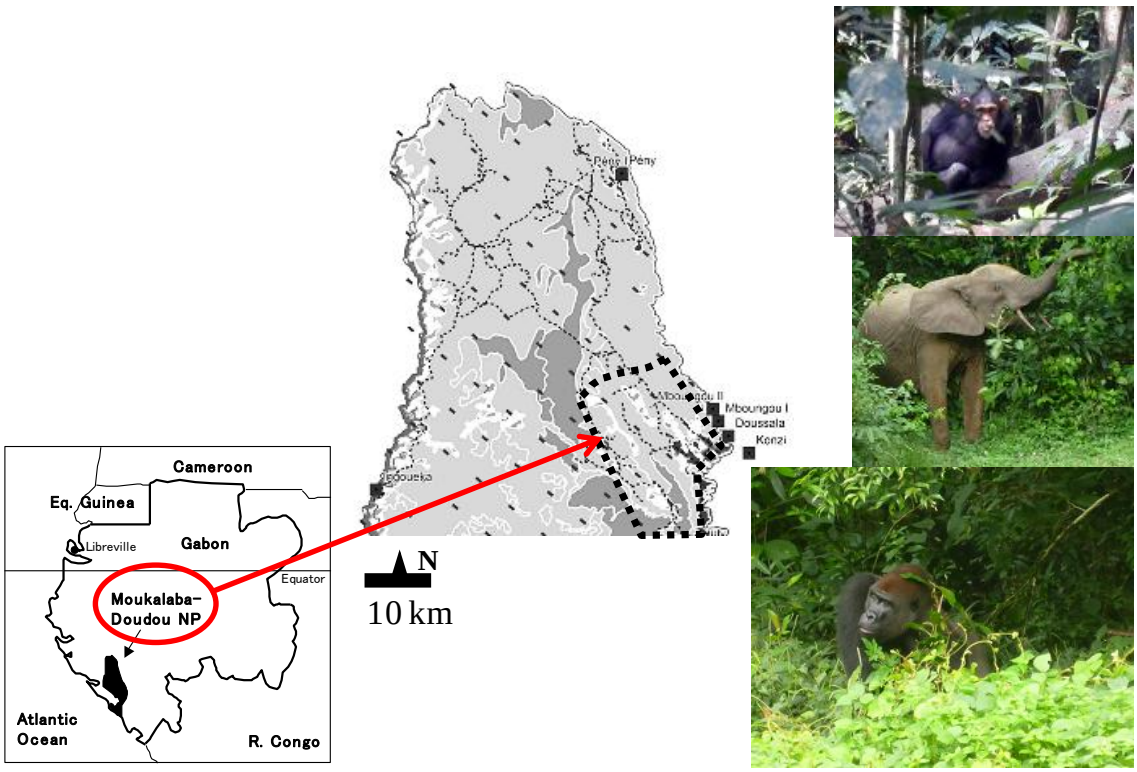


Fig. 1-1. Geographical information for Moukalaba-Doudou National Park in Gabon

Pictures show (top to bottom) a chimpanzee (juvenile male), a forest elephant (juvenile female), and a gorilla (solitary male).

Chapter 2

Bifidobacterium moukalabense sp. nov. isolated from the feces of wild western lowland gorilla (*Gorilla gorilla gorilla*) in Gabon

2-1 Introduction

Bifidobacteria are intestinal bacteria that are mostly regarded as human-associated bacteria, although they are distributed in a wide range of mammals (Mitsuoka and Kaneuchi 1977). It has been suggested that bifidobacteria are host- and animal-specific bacteria and are classified as ‘human type’, ‘animal type’ and others (Ventura *et al*. 2004). As shown in a recent study, it is important to isolate and identify novel bifidobacterium strains from various animals including humans to understand how they are distributed (Endo *et al*. 2012). We previously demonstrated that wild chimpanzees (*Pan troglodytes verus*) harbored bifidobacteria as a common component of their intestinal microbiota and these were human type, albeit a minor type among human bifidobacteria (Uenishi *et al.* 2007).

We successfully isolated *Bifidobacterium angulatum*-like bacteria from wild chimpanzees in Bossou, Guinea (Ushida *et al.* 2010). However, wild chimpanzees were suggested to possess non-human type bifidobacteria based on sequence analyses of the bifidobacterial 16S rRNA genes retrieved from feces of chimpanzees in Mahale, Tanzania (Ushida 2009). Unlike Chimpanzees in Bossou, which live close to villages with populations of about 3,000 and dense agricultural fields, chimpanzees in Mahale live in remote areas far from human agricultural activities. In our preliminary experiments, a bifidobacterial partial 16S rRNA gene similar to those of the chimpanzees in Mahale was retrieved from the feces of a wild lowland gorilla (*Gorilla*

gorilla gorilla) in Gabon. Thus, the presence of bifidobacteria associated with non-human great apes was suggested. The author reports here a novel species of the genus *Bifidobacterium* isolated from the feces of a wild lowland gorilla, central chimpanzees (*P. troglodytes troglodytes*) and a forest elephant (*Loxodonta africana cyclotis*) in Moukalaba-Doudou National Park (MDNP) in Gabon.

2-2 Materials and Methods

Fresh gorilla feces were collected in the forest of Boutiana in MDNP by chasing a group of gorillas (designated 'Group Gentil') in November 2010. This group included one silver back male, one black back male, six adult females, eleven juveniles or sub-adults and four babies. The distance between the gorillas and researchers did not allow for identifying which individual had defecated. Chimpanzees are not yet well habituated, but they sometimes allow the approaching researchers to collect fresh feces. Elephants are one of most dangerous animals in this study area, but their numerous fresh feces are relatively easily collected. Fresh feces of chimpanzees and a elephant were collected at opportunity in the same forest in November 2010.

Feces were collected in plastic bags with sterile tweezers to eliminate the portion contaminated with soil. After promptly returning to the base camp, a loopful of faecal specimen was inoculated on bifidobacteria selective (BS) agar plates. BS medium (Mitsuoka *et al*. 1965) was prepared with 58 g/L of BL agar medium (Nissui, Tokyo, Japan), 50 ml/L of defibrinated horse blood, 15 mg/L of sodium propionate (Wako, Osaka, Japan), 50 mg/L of paromomycin sulphate (Sigma-Aldrich Japan, Tokyo, Japan), 200 mg/L of fradiomycin sulphate (Wako) and 3 g/L of lithium chloride (Wako). The plates were preserved in air tight bags, each with an Anaeropouch®, (Mitsubishi Gas

Chemical Company, Tokyo, Japan) at a minimum temperature as possible. In the city, the bags were placed in a refrigerator and in the forest they were placed in a dark place until use.

After inoculation, the plates were preserved in plastic bags, each with an Anaeropouch®. After closing the bags, they were placed in a styrene foam box heated with hand warmers to maintain temperature as close to 37°C as possible, as in our previous study (Ushida *et al.* 2010). The bags were removed from the culture box when colony development was observed and stored as described above.

The developed colonies were purified in the same medium in the laboratory using several transfers to fresh media. Isolates were grown in GAM broth medium (Nissui, Tokyo, Japan) and subjected to DNA extraction. After bead disruption, DNA was extracted using a DNA isolation kit (Fujifilm, Tokyo, Japan).

Nearly complete (about 1500 bases) 16S rRNA gene sequences were determined as described previously (Tsukahara and Ushida 2002). Partial *hsp60* sequences were amplified by PCR using the primers H729 (5′-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGIACIACIAC-3′) and H730 (5′-AGCGGATAACAATTTCACACAGGAYKIYKITCICCRAAICCIGGIGCYTT-3′) (Sakamoto *et al.* 2010). Both amplicons were sequenced at Hokkaido System Science Co., Ltd. (Sapporo, Japan) by the dye-terminator method. Sequences of the closest observed relatives of the isolate were retrieved from public databases. Calculation of pair-wise 16S rRNA and *hsp60* gene sequence similarities was performed using MEGA version 5.05 (Tamura *et al*. 2011). Multiple sequence alignments were performed using the CLUSTAL W program (Thompson *et al*. 1994) and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987). Tree topology

was evaluated by a bootstrap analysis with 1000 replicates using CLUSTAL W. The minimum evolution with Kimura's two-parameter model (Kimura 1980) and the maximum-parsimony phylogenetic tree were inferred using MEGA version 5.05.

The extracted DNA was also subjected to nuclease treatment followed by HPLC analyses to determine G+C contents as described by Kitahara *et al.* (2005). Biochemical characteristics were evaluated using API 20A and API rapid ID 32A systems (Biomerieux; Paris, France) according to the manufacturer's instructions with incubation at 37°C in an anaerobic jar. A growth range test was performed using anaerobic GAM broth at 15, 25, 37, 40 and 45°C for 48 h. Major cellular fatty acid analysis was performed using Sherlock Microbial Identification System version 4.5 (Microbial Identification Inc, Newark, DE, USA) and their profiles were obtained using MIS Standard Libraries MOORE5.00 at Techno-Suruga Laboratory Co., Ltd. (Shizuoka, Japan).

2-3 Result and Discussion

Seventeen isolates are a gram staining positive rod with obligate anaerobic growth. Based on the 16S rRNA gene phylogeny, these isolate clearly belonged to the genus *Bifidobacterium* using neighbor-joining and minimum-evolution methods (Fig 2-1a, b). A maximum-parsimony analysis provided similar results (not shown). The type strains of *B. catenulatum* (98.3%) and *B. pseudocatenulatum* (98.1%) were the closest neighbors of these isolates, although these values were sufficiently low to propose a novel species of bifidobacteria without evidence from DNA–DNA hybridization (Stackebrandt and Ebers, 2006). The *hsp60* sequence of GG01^{T} presented the highest similarity to that of the type strain of *Bifidobacterium dentium* (92.2%), although the

phylogeny of its *hsp60* sequence presented that these isolates were a species clearly different from any known species (Fig 2-2).

We concluded that a novel species of the genus *Bifidobacterium* was isolated from a wild lowland gorilla in MDNP. The G+C content for the type strain of *B. moukalabense* was 60.1 mol% (Table 2-1). The biochemical characteristics of GG01^T are compared with those of *B. catenulatum* JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T and *B. dentium* JCM 1195^T (Table 2-2). Based on analyses with API 20A and API rapid ID 32A systems, these isolates possessed the same phenotypic pattern as those of *B. catenulatum* JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T and *B. dentium* JCM 1195^T, except for D-mannitol; cellobiose; melezitose; D-sorbitol; trehalose; leucyl glycine arylamidase; phenylalanine arylamidase; leucine arylamidase and alanine arylamidase. Anaerobic growth of strain GG01^T in GAM broth was observed at 25, 37 and 40°C (Table 2-1). The cellular fatty acid composition was: $C_{16:0}$ (42.59%); $C_{18:1}$ *ω9c* (15.64%); $C_{14:0}$ (13%); $C_{18:0}$ (9.65%); C_{19} cycloprop. 9, 10 (6.82%); $C_{18:1}$ *ω9c* DMA (2.96%); summed feature 10 (2.91%); $C_{19:0}$ cyclo 9, 10 DMA (1.75%); $C_{14:0}$ DMA (1.49%); $C_{12:0}$ (1.33%) and $C_{16:1}$ *ω9c* (1.21%) (Table 2-3). Palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) were the two most abundant fatty acids in our isolates, both of which are also major fatty acids in *B. catenulatum*, *B. pseudocatenulatum* and *B. dentium* (Morita *et al*., 2011). A particular components detected in our isolates were C_{19} cyclopropane fatty acid. In addition to its G+C content, differential biochemical characteristics and distinctive cellular fatty acid compositions are presented in Table 2-1.

Based on the results of this study, these isolates represent a novel species of the genus *Bifidobacterium*. We propose the species name *Bifidobacterium moukalabense* sp. nov. This novel species is considered to be a major *Bifidobacterium* in the intestine of

wild lowland gorillas, chimpanzees and elephants in MDNP.

This type strain has been included in the Japan Collection of Microorganisms (JCM) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) with the codes of JCM 18751^{T} and DSM 27321^{T}, respectively, for *B. moukalabense*.

2-4 Description of *Bifidobacterium moukalabense* sp. nov.

Bifidobacterium moukalabense [mou.ka.lab.en'se. N.L. neut. adj. moukalabense pertaining Moukalaba-Doudou National Park, from where the type strain was isolated].

Cells on the BL agar are rods of various shapes (0.6 μm wide and variable in length, primarily 2–4 μm) with rounded ends, often curved, swollen and branched. This isolate is gram staining positive, non-motile and obligatory anaerobic. Colonies on BS agar are 1–3 mm in diameter, brownish-red, opaque, convex and disc shaped after 48 h under anaerobic conditions at 37°C. The optimum growth temperature is 37°C. It produces acids from L-arabinose, glucose, lactose, sucrose, maltose, salicin, D-mannose and raffinose, but not from glycerol, D-mannitol, cellobiose, D-sorbitol, rhamnose or trehalose. It hydrolyses aesculin but not gelatin. Indole and urease are not produced. Using Rapid ID 32 A, the strain is positive for *α*- and *β*-galactosidase, *α*- and *β*-glucosidase, *α*-arabinosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase, but negative for arginine dihydorase, *β*-galactosidase-6-phosphate, *β*-glucuronidase, *N*-acaetyl-*β*-glucosamidase, glutamic acid decarboxylase, *α*-fucosidase, alkaline

phosphatase, pyroglutamic acid arylamidase, alanine arylamidase and glutamyl glutamic acid arylamidase. The major fatty acids are $C_{16:0}$, $C_{18:1}\omega 9c$ and $C_{14:0}$. The DNA G+C content of the type strain is 60.1 mol%.

Table 2-1. Characteristics of strain GG01^T and those with closest phylogenetic neighbors*

Characteristic	*B. moukalabense* GG01^T	*B. catenulatum* JCM 1194^T	*B. pseudocatenulatum* JCM 1200^T	*B. dentium* JCM 1195^T
Acid production from (API 20A)				
D-Mannitol	-	-	-	+
Cellobiose	-	+	+	+
Melezitose	+	+	+	-
D-Sorbitol	-	+	+	+
Trehalose	-	+	+	+
Rapid ID 32A results				
Leucyl glycine arylamidase	+	-	-	-
Phenylalanine arylamida	+	+	+	-
Leucine arylamidase	+	+	+	-
Alanine arylamidase	-	-	+	-
Distinctive fatty acid(s)§	C_{19} cycloprop. 9,10, $C_{19:0}$ cyclo 9, 10 DMA	$C_{16:0}$ $\omega 7c$ DMA, $C_{18:0}$ 12-OH[a]†	$C_{10:0}$[a]	-
Temperature range for growth (ºC)	25-40	25-40	25-45	25-40
DNA G+C content (mol%)	60.1	54.7[b]	57.5[c]	61.2[b]

*Adapted and modified from Table 1 in Tsuchida *et al*.2014a.

§ Fatty acids detected only in the strain indicated. Details are shown in Table 2-3. Data were obtained in this study unless otherwise indicated.

†Data taken from: a, Morita *et al*. (2011); b, Scardovi *et al*. (1974); c, Scardovi *et al*. (1979).

Table 2-2. Phenotypic characteristics for strain GG01^T of *Bifidobacterium moukalabense* sp. nov. and their closest phylogenetic neighbors*

Characteristic	*B. moukalabense* GG01^T	*B. catenulatum* JCM 1194^T	*B. pseudocatenulatum* JCM 1200^T	*B. dentium* JCM1195^T
API 20A results				
D-Mannitol	-	-	-	+
Cellobiose	-	+	+	+
Melezitose	+	+	+	-
D-Sorbitol	-	+	+	-
Trehalose	-	+	+	+
All strains positive	Aesculin hydrolysis, Glucose, Lactose, Sucrose, Maltose, Salicin, D-Xylose, L-Arabinose, D-Mannose, Raffinose			
All strains negative	Indole production, Urease, Gelatin hydrolysis, Glycerol, L-Rhamnose			
Rapid ID 32A results				
Leucyl glycine arylamidase	+	-	-	-
Phenylalanine arylamidase	+	+	+	-
Leucine arylamidase	+	+	+	-
Alanine arylamidase	-	-	+	-
All strains positive	α-Galactosidase, β-Galactosidase, α-Glucosidase, β-Glucosidase, α-Arabinosidase, Mannose, Raffinose, Arginine arylamidase, Proline arylamidase, Tyrosine arylamidase, Glycine arylamidase, Histidine arylamidase, Serine arylamidase			
All strains negative	Urease, Arginine dihydolase, β-Galactosidase, β-Glucuronidase, N-Acetyl-β-glucosaminidase, Glutamic acid decarboxylase, α-Fucosidase, Nitrate reduction, Indole production, Alkaline phosphatase, Pyroglutamic acid arylamidase, Glutamyl glutamic acid arylamidase			

*Adapted and modified from Table S1 in Tsuchida *et al*.2014a.

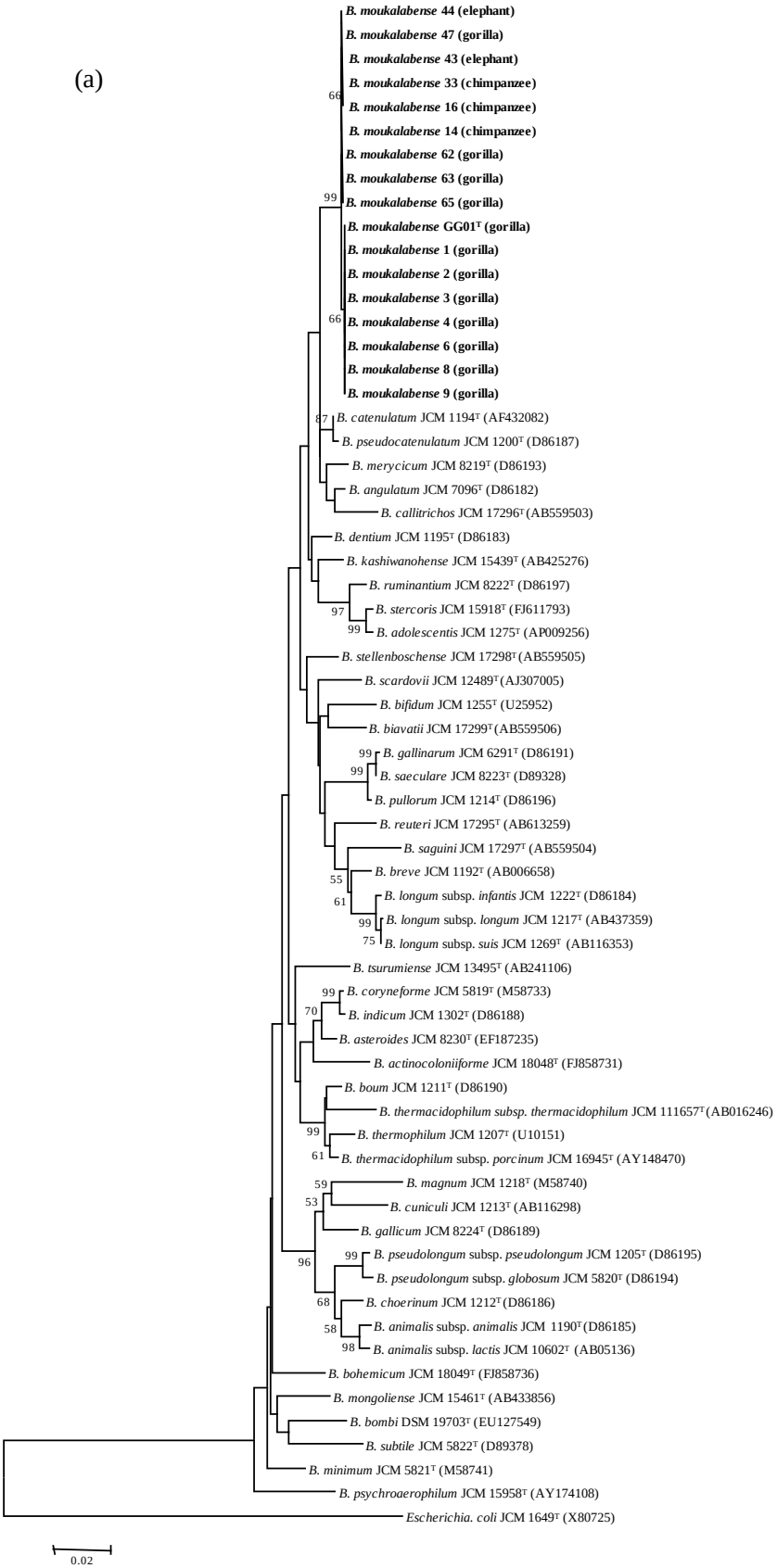
Table 2-3. Cellular fatty acid compositions of strain GG01^T and their closest phylogenetic neighbors*

Fatty acid	*B. moukalabense* GG01^T	*B. catenulatum* JCM1194^T†	*B. pseudocatenulatum* JCM1200^T†	*B. dentium* JCM1195^T†
Saturated strainght-chain:				
$C_{10:0}$	-	-	1.2	-
$C_{12:0}$	1.33	1.3	2.9	1.2
$C_{14:0}$	13	5.3	10.5	5.1
$C_{16:0}$	42.59	25.7	13.7	25.8
$C_{18:0}$	9.65	3.3	2.3	6.9
$C_{14:0}$ DMA	1.49	3.5	2.7	2.9
$C_{19:0}$ cyclo 9,10 DMA	1.75	-	-	-
Unsaturated strainght-chain:				
$C_{16:1}$ *ω7c*	-	1.8	3.9	1.1
$C_{16:1}$ *ω7c* DMA	-	1.1	-	-
$C_{16:1}$ *ω9c*	1.21	2.2	2.2	1.8
$C_{18:1}$ *ω9c*	15.64	18.3	33.6	22.8
$C_{18:1}$ *ω9c* DMA	2.96	23.3	12.2	23.2
Hydroxy				
$C_{18:0}$ 12-OH	-	1.5	-	-
Cyclopropane acid				
C_{19} cycloprop. 9,10	6.82	-	-	-
Summed feature*				
10	2.91	-	-	-

*Adapted and modified from Table S2 in Tsuchida *et al.*2014a.

Data are percentages of total fatty acids; values ≥ 1% are shown. ECL, Equivalent chain-length. *Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 10 consisted C $_{18:1}$*ω7c* and an unknown fatty acid ECL 17.834. †Data taken from: Morita *et al*. (2011)

(a)


B. moukalabense 44 (elephant)
B. moukalabense 47 (gorilla)
B. moukalabense 43 (elephant)
B. moukalabense 33 (chimpanzee)
B. moukalabense 16 (chimpanzee)
B. moukalabense 14 (chimpanzee)
B. moukalabense 62 (gorilla)
B. moukalabense 63 (gorilla)
B. moukalabense 65 (gorilla)
B. moukalabense GG01T (gorilla)
B. moukalabense 1 (gorilla)
B. moukalabense 2 (gorilla)
B. moukalabense 3 (gorilla)
B. moukalabense 4 (gorilla)
B. moukalabense 6 (gorilla)
B. moukalabense 8 (gorilla)
B. moukalabense 9 (gorilla)
B. catenulatum JCM 1194T (AF432082)
B. pseudocatenulatum JCM 1200T (D86187)
B. merycicum JCM 8219T (D86193)
B. angulatum JCM 7096T (D86182)
B. callitrichos JCM 17296T (AB559503)
B. dentium JCM 1195T (D86183)
B. kashiwanohense JCM 15439T (AB425276)
B. ruminantium JCM 8222T (D86197)
B. stercoris JCM 15918T (FJ611793)
B. adolescentis JCM 1275T (AP009256)
B. stellenboschense JCM 17298T (AB559505)
B. scardovii JCM 12489T (AJ307005)
B. bifidum JCM 1255T (U25952)
B. biavatii JCM 17299T (AB559506)
B. gallinarum JCM 6291T (D86191)
B. saeculare JCM 8223T (D89328)
B. pullorum JCM 1214T (D86196)
B. reuteri JCM 17295T (AB613259)
B. saguini JCM 17297T (AB559504)
B. breve JCM 1192T (AB006658)
B. longum subsp. infantis JCM 1222T (D86184)
B. longum subsp. longum JCM 1217T (AB437359)
B. longum subsp. suis JCM 1269T (AB116353)
B. tsurumiense JCM 13495T (AB241106)
B. coryneforme JCM 5819T (M58733)
B. indicum JCM 1302T (D86188)
B. asteroides JCM 8230T (EF187235)
B. actinocoloniiforme JCM 18048T (FJ858731)
B. boum JCM 1211T (D86190)
B. thermacidophilum subsp. thermacidophilum JCM 11165T (AB016246)
B. thermophilum JCM 1207T (U10151)
B. thermacidophilum subsp. porcinum JCM 16945T (AY148470)
B. magnum JCM 1218T (M58740)
B. cuniculi JCM 1213T (AB116298)
B. gallicum JCM 8224T (D86189)
B. pseudolongum subsp. pseudolongum JCM 1205T (D86195)
B. pseudolongum subsp. globosum JCM 5820T (D86194)
B. choerinum JCM 1212T (D86186)
B. animalis subsp. animalis JCM 1190T (D86185)
B. animalis subsp. lactis JCM 10602T (AB05136)
B. bohemicum JCM 18049T (FJ858736)
B. mongoliense JCM 15461T (AB433856)
B. bombi DSM 19703T (EU127549)
B. subtile JCM 5822T (D89378)
B. minimum JCM 5821T (M58741)
B. psychroaerophilum JCM 15958T (AY174108)
Escherichia. coli JCM 1649T (X80725)
0.02

(b)

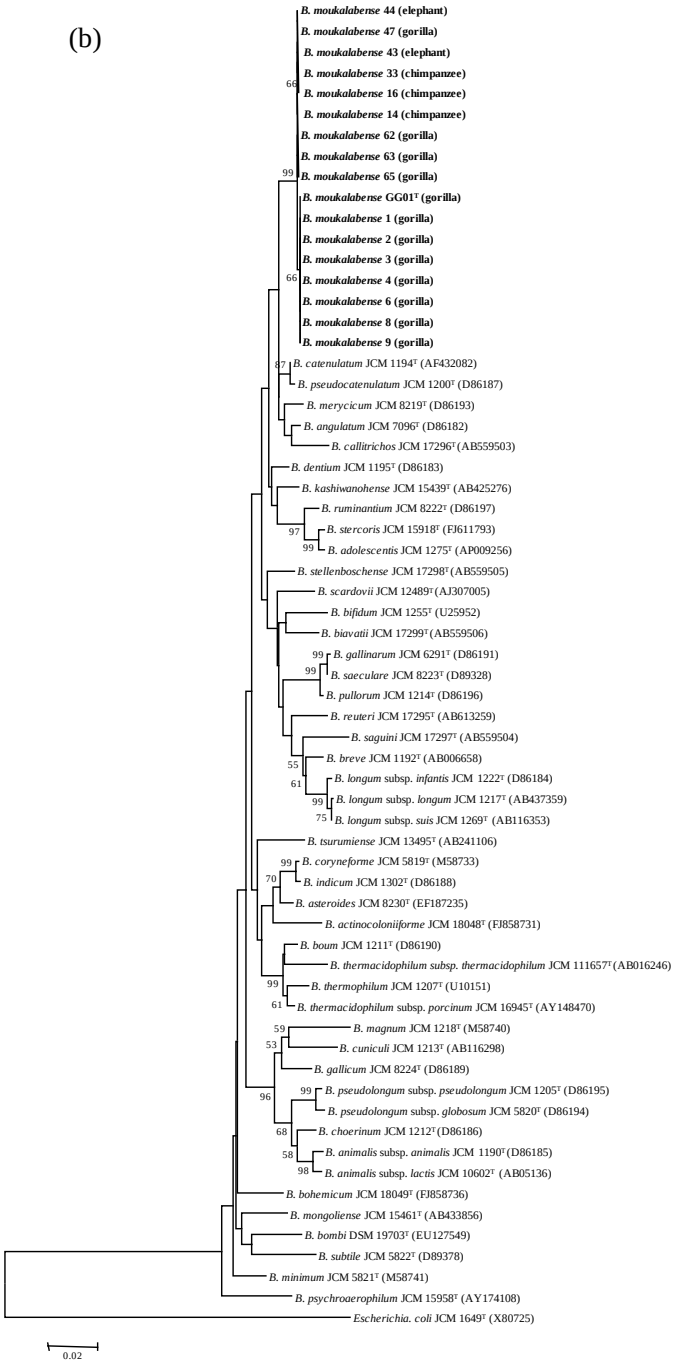


Fig. 2-1. Neighbor-joining (a) and minimum-evolution (b) phylogenetic trees based on 16S rRNA gene sequence showing relationships between *B. moukalabense* and members of related *Bifidobacterium* species. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. Bars represent 0.02 (a) and (b) substitutions per nucleotide position.

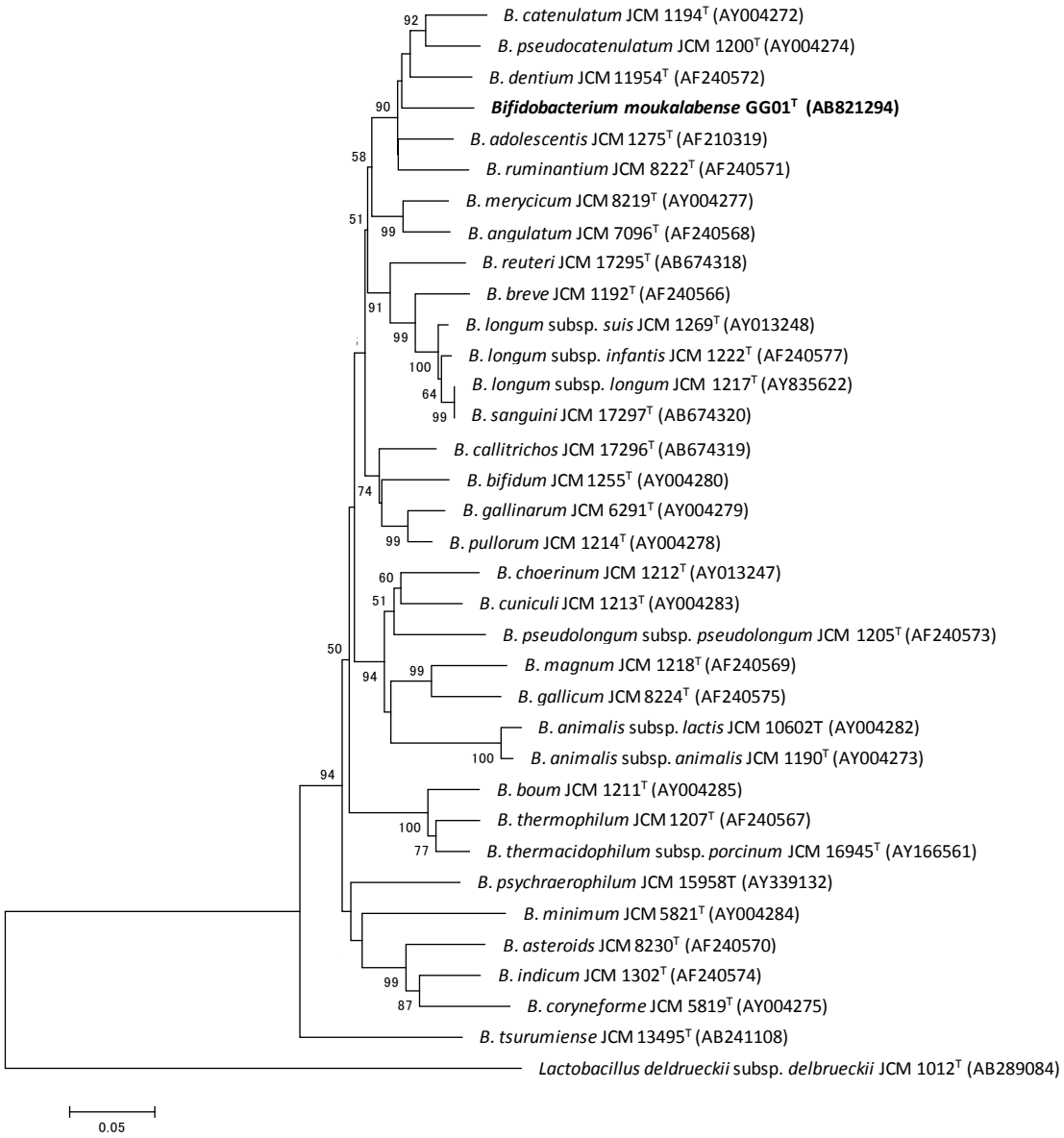


Fig. 2-2. Neighbor-joining phylogenetic tree based on *hsp60* gene sequence showing relationships between strain GG01^T and members of related *Bifidobacterium* species. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. Bars represent 0.05 substitutions per nucleotide position. *Adapted and modified from Fig 2 in Tsuchida *et al*.2014a.

Chapter 3

Lactobacillus gorillae sp. nov. isolated from the feces of captive and wild western lowland gorillas (*Gorilla gorilla gorilla*)

3-1 Introduction

Lactobacilli are well studied lactic acid bacteria and isolated from various habitats such as fermented food, animal intestine and insect intestine (Irisawa *et al.*, 2014, Killer *et al.*, 2014, Olofsson *et al.*, 2014). Furthermore now comprised of over 200 recognized species (Euzéby, 2014). Lactobacilli are commonly found in the gastrointestinal microbiota of a wide range of mammals (Mitsuoka & Kaneuchi, 1977) and each animal species hosts different species of *Lactobacillus* in their gastrointestinal tracts (Endo *et al*., 2010). However, previous studies have mostly focused on the microbiota of humans, livestock or captured animals. Therefore, we have focused on the gastrointestinal microbiota of wild animals (Tsuchida *et al.*, 2014). In this study, we attempted to isolate and identify novel *Lactobacillus* strains from various wild animals to determine the host specific distributions of *Lactobacilli* and factors that affected their distributions.

During a study on Lactobacilli in primates, particularly those in the wild, we isolated four previously unidentified strains. Therefore, we report here a novel species of the genus *Lactobacillus* that was isolated from the feces of captive and wild western lowland gorillas.

3-2 Materials and Methods

Fresh gorilla feces were collected from two captive gorillas (adult female and

adult male) at the Kyoto City Zoo in June 2013 and from one wild gorilla (adult female or male) in the Boutiana forest in the Moukalaba-Doudou National Park (MDNP), Gabon. Collected feces taken at the Kyoto City Zoo were identified on the basis of the volume and form. In MDNP, we collected feces by chasing a group of gorillas (designated 'Group Gentil') in November 2009. This group included one silver back male, one black back male, six adult females, eleven juveniles or sub-adults and four babies. Although the distance between the gorillas and researchers did not allow identifying which individual had defecated, the volume of feces was enough visible to distinguish feces of the adult from the juvenile.

The feces of captive and wild western lowland gorillas were collected in plastic bags with sterile tweezers to eliminate that portion contaminated with soil. A loopful of a faecal specimen was inoculated on LBS (BBL) agar plates. These plates were incubated at 37°C in an incubator for the feces of captured gorillas. Anaerobiosis was maintained with Anaeropouch® (Mitsubishi Gas Chemical Company, Tokyo, Japan). In the field of MDNP, the plates were kept as close to 37°C as possible under anaerobic conditions using an Anaeropouch® as indicated in our previous study (Tsuchida *et al*., 2014).

The developed colonies were purified in the same medium in the laboratory using several transfers to fresh media. Isolates were grown in GAM broth (Nissui, Tokyo, Japan) and used for DNA extraction. After bead-disruption, DNA was extracted using a DNA isolation kit (QuickGene-Mini80; Fujifilm, Tokyo, Japan).

Nearly complete (approximately 1,500 bases) 16S rRNA gene sequences were determined as previously described (Tsukahara & Ushida, 2002). Partial *pheS* sequences were amplified by PCR using the primers *pheS*-21F

(5′-CAYCCNGCHCGYGAYATGC-3′) and *pheS*-22R (5′-CCWARVCCRAARGCAAARCC-3′) (Naser *et al.*, 2005). Both amplicons were sequenced at Hokkaido System Science Co., Ltd. (Sapporo, Japan) using the dye-terminator method. The sequences of the closest observed relatives of an isolate were retrieved from public databases. Calculations for pair-wise 16S rRNA and partial *pheS* gene sequence similarities were made using MEGA version 6.06 (Tamura *et al*., 2013). Multiple sequence alignments were prepared using the CLUSTAL W program (Thompson *et al*., 1994) and phylogenetic trees were constructed using the neighbor-joining method (Saitou & Nei, 1987). Tree topology was evaluated with a bootstrap analysis with 1000 replicates using CLUSTAL W. The minimum -evolution with Kimura's two-parameter model (Kimura, 1980) and the maximum -likelihood phylogenetic tree were inferred using MEGA version 6.06.

DNA–DNA hybridization was performed among strains KZ01^{T}, KZ02, KZ03, GG02 and *L. fermentum* JCM 1173^{T} according to a published method by using photobiotin and microplates (Ezaki *et al*., 1989).

An automated ribotyping device, RiboPrinter ®microbial characterization system (Qualicon), was used for ribotyping according to the manufacturer's instructions, with *Eco*RI used as the restriction enzyme. Ribopatterns were analysed by BioNumerics version 2.5 software (Applied Maths) and were compared by Pearson similarity coefficient analysis and the unweighted pair group method using arithmetic average (UPGMA) algorithm.

Extracted DNA was also subjected to nuclease treatment followed by HPLC analyses to determine G + C contents, as described by Kitahara *et al.* (2005). Biochemical characteristics were evaluated using API 50 CH and API ZYM systems

(Biomerieux; Paris, France), according to the manufacturer's instructions. A growth range test was done using LBS broth at 15°C and 45°C for 48 h under anaerobic conditions. Tolerance to NaCl was examined in LBS broth containing 4.0, 6.5, 8.0 and 10.0 % (w/v) NaCl after incubation for 7 days at 37°C. The proportions of D- and L-lactate were determined enzymically using DL-lactate test kit (Megazyme, Ireland). Cellular fatty acid profiles were determined using a Microbial Identification System (Microbial ID; MIDI). Cell wall peptidoglycans were prepared by the method of Kawamoto *et al*. (1981) and the amino acid contents in peptidoglycans were determined as described by Ahmed *et al*. (2014).

This study was conducted non-invasively. Feces were collected in Kyoto City Zoo during routine litter clean up with the permission. Feces of wild gorilla were collected with a research permission from National Center of Scientific Research and Technology, Ministry of Higher Education, Scientific Research and Technological Development , Gabonese Republic under the contract between Kyoto University and this center established since 2009.

3-3 Result and Discussion

Fifteen isolates from captive and wild western lowland gorillas were a gram - staining positive rod with facultative anaerobic growth. Based on 16S rRNA gene phylogeny, these isolates had identical 16S rRNA gene sequences and, using neighbor-joining analysis, clearly belonged to the *Lactobacillus reuteri* phylogenetic group (Fig. 3-1). Maximum likelihood analysis and minimum evolution analysis provided similar results (data not shown). *L. fermentum* JCM 1173^{T} (96.6%) was the closest neighbor to type strain $KZ01^{T}$. Strains $KZ01^{T}$, KZ02 and GG02 had identical

partial *pheS* gene sequences. The sequence of KZ01^T exhibited the highest similarity to that of *L. fermentum* JCM 1173^T (81.2%), although the phylogeny of these partial *pheS* gene sequences showed that these isolates were from a species that was clearly different from any known species (Fig. 3-2).

The DNA–DNA relatedness values between strains KZ01^T, KZ02, KZ03, GG02 and *L. fermentum* JCM 1173^T were very low (11%–22%). In contract, strains KZ01^T, KZ02, KZ03 and GG02 showed a high value of DNA–DNA relatedness (83–92%) (Table 3-3).

Ribotyping with the RiboPrinter system was used to investigate the relationships between strains KZ01^T, KZ02, KZ03, GG02 and *L. fermentum* JCM 1173^T (Fig. 3-3). A dendrogram based on ribotyping patterns showed a single cluster for four stains of *L. gorillae*. In addition, KZ01^T and KZ02 had similar ribotyping patterns and KZ03 and GG02 also had similar ribotyping patterns.

Therefore, we concluded that a novel species of the genus *Lactobacillus* had been isolated from captured and wild western lowland gorillas and gave this the species name *L. gorillae*.

The G + C contents for *L. gorillae* were 50.7–52.3 mol%, which was within the ranges reported for the genus *Lactobacillus* (Kandler and Weiss 1986) (Table 3-1). The phenotypic characteristics of the four strains of *L. gorillae* were compared with those of *L. fermentum* JCM 1173^T and these details are shown in Table 3-1.

The cellular fatty acid compositions of the four strains of *L. gorillae* included saturated, unsaturated, cyclopropane and summed feature fatty acids (Table 3-2). Therefore, the major cellular fatty acids of *L. gorillae* were $C_{16:0}$, $C_{18:1}$ *ω9c* and $C_{19:1}$ cyclo 9,10. Cleary different cellular fatty acid compositions from that of *L. fermentum*

JCM 1173^T were $C_{14:0}$ and $C_{18:0}$ (Table 3-1).

The four strains of *L. gorillae* and *L. fermentum* JCM 1173^T contained ornithine, glutamic acid and alanine. The inter-peptide bridges of peptidoglycans comprised L-ornithine and D-glutamic acid as diagnostic amino acids, which corresponded to cell wall peptidoglycan type A4β (Schleifer and Kandler 1972).

Based on our analyses with the API 50 CH and API ZYM systems, *L. gorillae* had the same phenotypic pattern as that of *L. fermentum* JCM 1173^T, except for cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and α-glucosidase. Acid production from D-xylose, arbutin, esculin, salicin, cellobiose, lactose and trehalose showed some variations between the four strains of *L. gorillae*. Anaerobic growth of *L. gorillae* on LBS agar was not observed at 15°C and 45°C (Table 3-1). Four strains of *L. gorillae* grew in the presence of 4.0 –6.5% NaCl and only KZ01T, KZ02 and KZ03 grew in the presence of 8.0% NaCl. Growths of these isolates were not in the presence of 10.0% NaCl (Table 3-1). They were heterofermentative. Both D- and L-lactic acids were produced. In addition to the G + C contents, differential biochemical characteristics and distinctive cellular fatty acid compositions are shown in Table 3-1.

Based on the results of this study in accordance with minimal standards for description of new taxa of genera Lactobacillus by Mattarelli *et al.* (2014), 15 strains from captive and wild western lowland gorillas represent a novel species of the genus *Lactobacillus*. We propose the species name *Lactobacillus gorillae* sp. nov. We consider this novel species to be a major *Lactobacillus* in the intestines of western lowland gorillas.

Strain KZ01^T has been included in the Japan Collection of Microorganisms (JCM) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

(DSMZ) with the codes JCM 19575^{T} and DSM 28356^{T}, respectively, for *L. gorillae*.

3-4 Description of *Lactobacillus gorillae* sp. nov.

Lactobacillus gorillae [go.rillae. N.L. n. Gorilla, zoological genus name of the western lowland gorilla; N. L. gen. n. gorillae, of the western lowland gorilla].

Cells on LBS agar are rod-shaped (1 μm wide and variable in length, primarily 3–8 μm) with rounded ends and are non-spore forming. These isolates are gram stain positive, non-motile, facultative anaerobes. Colonies on LBS agar are 1–2 mm in diameter, white, convex and smooth disc shaped after 48 h at 37°C under anaerobic conditions. They are heterofermentative. Both D- and L-lactic acids are produced. The type strain grows in the presence of 4.0 –8.0 % NaCl but not in the presence 10.0% NaCl. They produce acids from ribose, galactose, glucose, fructose, mannose, maltose, melibiose, sucrose and raffinose but not from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, *β*-methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, *α*-methyl-D-mannoside, *α*-methyl-D-glucoside, *N*-acetyl-glucosamine, amygdalin, inulin, melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate or 5-keto-gluconate. Acid production from D-xylose, arbutin, esculin, salicin, cellobiose, lactose, trehalose and gluconate is variable. Using the API ZYM system, these strains are positive for esterase, esterase lipase, leucine arylamidase, valin arylamidase, phosphatase, *α*-galactosidase and *β*-galactosidase but are negative for alkaline phosphatase, lipase, cystine arylamidase, trypsin, *α*-chymotrypsin, naphthol-AS-BI-phosphohydrolase, *β*-glucuronidase, *α*-gulcosidase, *β*-gulcosidase, *N*-acetyl-*β*-glucosamidase, *α*-mannosidase and *α*-fucosidase. Their cell

wall peptidoglycan type is A4β (L-Orn-D-Asp). Their major fatty acids are $C_{16:0}$, $C_{18:1}$ *ω9c* and $C_{19:1}$ cyclo 9,10. The DNA G + C content of the type strain is 50.7 mol%.

Table 3-1. Characteristics of *L. gorillae* sp. nov. and those of the closest phylogenetic neighbor

Characteristic	*L. gorillae* KZ01^T	*L. gorillae* KZ02	*L. gorillae* KZ03	*L. gorillae* GG02	*L. fermentum* JCM1173^T
Acid production from (API 50CH):					
D-xylose	-	-	+	+	-
Arbutin	-	-	w	w	-
Esculin	+	-	+	+	-
Salicin	-	-	-	w	-
Cellobiose	-	-	w	w	-
Lactose	-	-	+	w	+
Trehalose	-	-	+	+	-
API ZYM results:					
Cystine arylamidase	-	-	-	-	+
Naphthol-AS-BI-phosphohydrolase	-	-	-	-	+
α-galactosidase	+	+	+	+	-
α-glucosidase	-	-	-	-	+
Distinctive cellar fatty acid(s)§	$C_{14:0}$, $C_{18:0}$	$C_{14:0}$, $C_{18:0}$	$C_{14:0}$, $C_{18:0}$	$C_{14:0}$, $C_{18:0}$	-
Growth at 15/45 °C	-/-	-/-	-/-	-/-	w/w
Growth in Nacl					
6.5%	+	+	+	+	-
8%	+	+	+	-	-
DNA G+C content (mol%)	50.7	51.3	51.2	52.3	†52-54

§ Fatty acids detected only in the particular strain are indicated. Details are shown in Table S1. Data were obtained during this study unless otherwise indicated.

†Data from: Dellaglio *et al*. 2004.

*Adapted and modified from Table 1 in Tsuchida *et al*.2014c.

Table 3-2. Cellular fatty acid compositions of the *L. gorillae* sp. nov. strains and those of the closest phylogenetic neighbor

Fatty acid	*L. gorillae* KZ01^T	*L. gorillae* KZ02	*L. gorillae* KZ03	*L. gorillae* GG02	*L. fermentum* JCM1173^T
Saturated strainght-chain:					
$C_{14:0}$	1.62	1.79	2.35	3.59	-
$C_{16:0}$	25.95	24.88	25.23	26.1	27.83
$C_{18:0}$	2.51	2.67	3.17	3.08	-
Unsaturated strainght-chain:					
$C_{18:1}$ *ω9c*	27.44	28.49	29.1	28.78	13.61
$C_{18:2}$ *ω6,9c*	-	1.1	-	-	-
Cyclopropane					
$C_{19:1}$ cyclo 9,10	16.62	22.4	23.01	22.97	11.96
$C_{19:1}$ cyclo 11,12	13.22	10.61	9.2	8.42	25.99
Summed feature*					
10	9.57	8.05	7.01	6.17	20.6

Data are percentages of total fatty acids; values ≥ 1% are shown.

*Summed features represent groups of two or three fatty acids that could not be separated using the Microbial Identification System. Summed feature 10 consisted of $C_{18:1}$*ω*11*c*/*ω*9*t*/*ω*6*t*. *Adapted and modified from Table 2 in Tsuchida *et al*.2014c.

Table 3-3. DNA－DNA hybridization values for *Lactobacillus gorillae* sp. nov. and *Lactobacillus fermentum*

		Probe				
		L. gorillae KZ01^T	*L. gorillae* KZ02	*L. gorillae* KZ03	*L. gorillae* GG02	*L. fermentum* JCM 1173^T
DNA	*L. gorillae* KZ01^T	100	108	105	114	22
	L. gorillae KZ02	83	100	92	102	15
	L. gorillae KZ03	92	100	100	105	17
	L. gorillae GG02	85	88	86	100	11
	L. fermentum JCM 1173^T	40	17	35	29	100

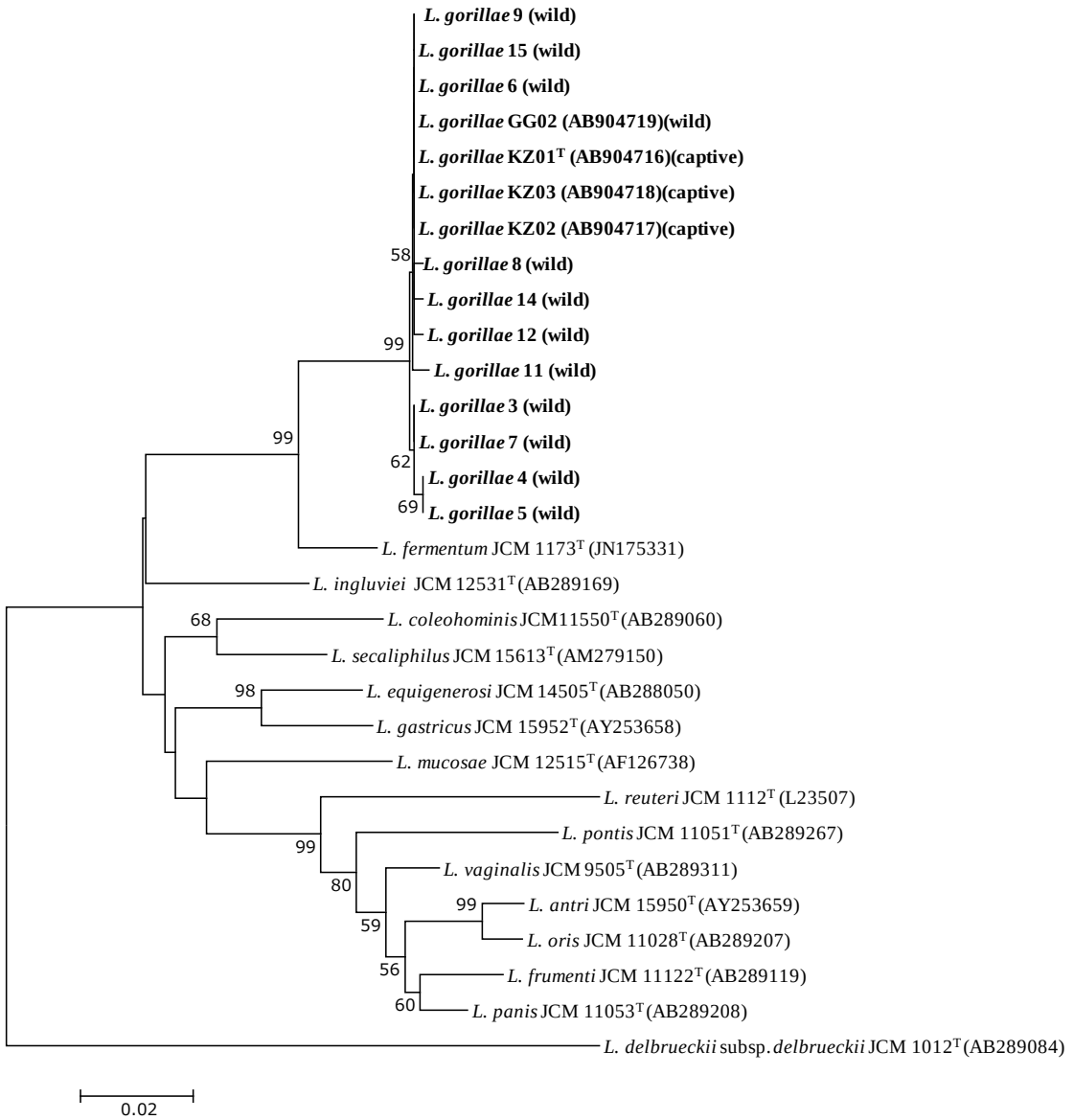


Fig. 3-1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between *L. gorillae* sp. nov. and members of related *Lactobacillus* species. Bootstrap values (>50%) based on 1,000 replicates are shown at branch nodes. Bars represent 0.02 substitutions per nucleotide position.

*Adapted and modified from Fig 1 in Tsuchida *et al*.2014c.

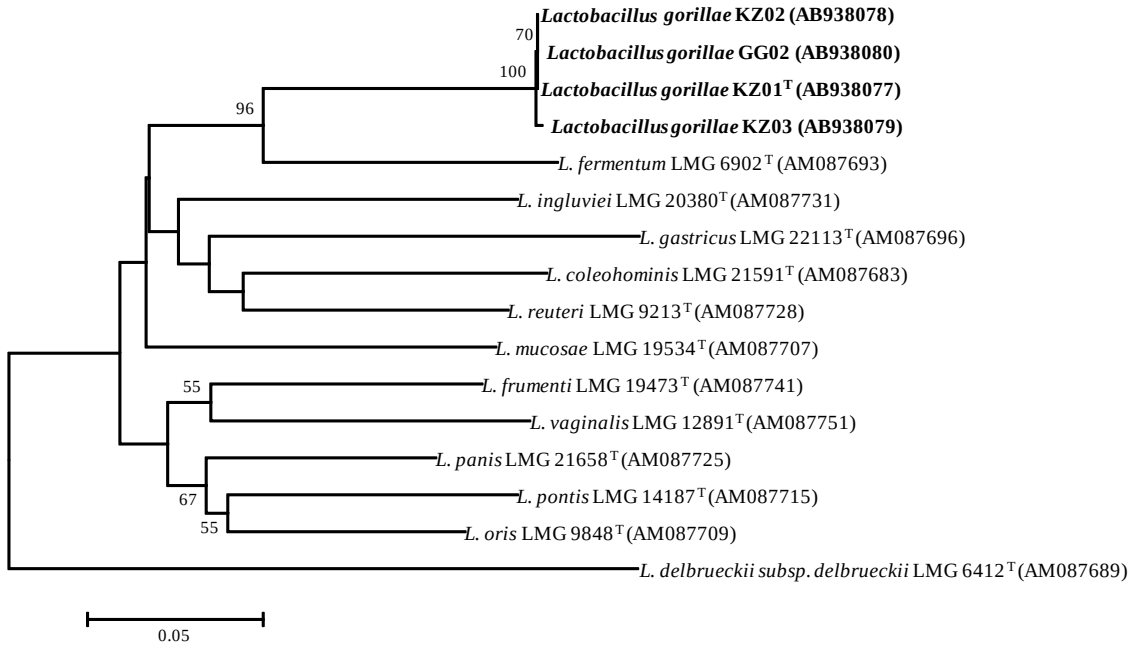


Fig. 3-2. Neighbor-joining phylogenetic tree based on partial *pheS* gene sequences showing the relationships between strains KZ01^T, KZ02, KZ03, GG02 and members of related *Bifidobacterium* species. Bootstrap values (>50%) based on 1,000 replicates are shown at branch nodes. Bars represent 0.05 substitutions per nucleotide position.

*Adapted and modified from Fig 2 in Tsuchida *et al*.2014c.

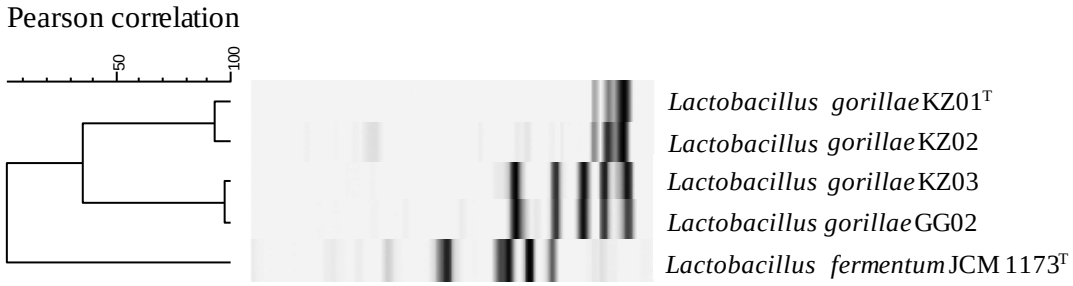


Fig. 3-3. Dendrogram illustrating the relatedness of the ribotyping patterns of strains KZ01^T, KZ02, KZ03, GG02 and *L. fermentum* JCM 1173^T. This dendrogram was analysed using Pearson similarity coefficient analysis and the UPGMA algorithm.

*Adapted and modified from Fig 3 in Tsuchida *et al*.2014c.

Chapter 4

Characterization of intestinal bacterial communities of western lowland gorillas (*Gorilla gorilla gorilla*), central chimpanzees (*Pan troglodytes troglodytes*), and a forest elephant (*Loxodonta africana cyclotis*) living in Moukalaba-Doudou National Park in Gabon

4-1 Introduction

Intestinal microbiota in the mammal develop a complex ecosystem of vast diversity after birth. For example, humans and mice, as experimental animals, possess at least 1,000 and 400 phylogenetically different bacteria, respectively (Hooper 2004, Qin *et al*. 2010). It has been speculated that such an ecosystem may develop in a host-specific manner (Ley *et al*. 2008). This was evidenced by the earlier studies done by Mitsuoka and Kaneuchi (1977) in which each animal species possesses a particular composition of microbiota. The interaction between bacteria and the intestinal mucosa of the host explains the selection of bacteria to some extent (Yamamoto *et al*. 1996, Kelly *et al*. 2005, Uchida *et al*. 2006). The feeding behavior of the hosts further selects the bacteria that can reside in their intestine. In this context, there may be clear differences in the composition of microbiota between herbivores and carnivores (Mitsuoka and Kaneuchi 1977, Ley *et al*. 2008, Endo *et al*. 2010).

Intestinal microbiota have been intensively studied in humans, model animals such as rats or mice, and livestock, including some pet animals, due to their economic importance (Zhou *et al*. 2007, Dowd *et al*. 2008, Turnbaugh *et al*. 2008, Hill *et al*. 2010, Handl *et al*. 2011). Indeed, many studies are concerned with gut microbiota in order to characterize its particular relationship to metabolic disorders and chronic diseases in

view of the loss of health-promoting indigenous bacteria (Kellermayer 2013, Andoh *et al*. 2007, Wen *et al*. 2008). Such protective microbiota should have a particular relationship with their host, and such a relationship can be explained by the concept of co-evolution between the host and its intestinal microbiota (Amato 2014). Comprehensive analyses on the microbiota of wild animals have been out of focus for such nutritional and pathological studies. Accordingly, there are only a few studies elucidating on the microbiota of wild animals except for those of the great apes under captivity (Kisidayová *et al*. 2009, Vlčková *et al*. 2012). We believe that the surveys on the intestinal microbiota of wild animals have tremendous importance for the understanding of the co-evolution between the host and its intestinal microbiota. Our previous study reveals that the fecal microbiota of wild chimpanzees (*P. troglodytes verus*) were clearly different from those under captivity with some particular influences from human-associated bacteria (Uenishi *et al*. 2007, Ushida 2009). Studies on captive animals may have limitations in revealing the original composition of intestinal microbiota of the target animals.

Our previous study adopted 16S rDNA-based temperature gradient gel electrophoresis (TGGE), which enables us to analyze bacteria of top 20-level abundance (Uenishi *et al*. 2007). Recent developments in sequencing technology can characterize the individual differences of human microbiota by deep sequencing (Wu *et al*. 2010). Therefore, we decided to study the intestinal microbiota of gorillas, chimpanzees, and an elephant in the wild by using pyrosequencing analysis for a more precise understanding of their intestinal microbiota. We have selected western lowland gorillas (*Gorilla gorilla gorilla*) and central chimpanzees (*P. troglodytes troglodytes*) in Moukalaba-Doudou National Park (MDNP) in Gabon as targets because they are

sympatric in depending on nearly the same food variety, with the exception of temporary ingestion of insects, which is one of preferred food for chimpanzees in general (Tutin and Fernandez 1993, Yamagiwa and Basabose 2006). As mentioned above, the food habits of the hosts select the intestinal bacteria. It is important to compare the intestinal bacteria between sympatric apes, gorillas, and chimpanzees, which reflect their adaptation to a particular food habit. In this context, we are also interested in intestinal microbiota of forest elephants (*Loxodonta africana cyclotis*), which are the major herbivore animals in the study area that forage grass, leaves, and fruits. In general terms, the chemical components of their food seem to be similar to those of the food of gorillas and chimpanzees.

This is the first report on the comparison of the intestinal microbiota of sympatric wild gorillas and wild chimpanzees. This is also the first bacteriological study on the wild forest elephant.

4-2 Materials and Methods

4-2-1 Study site

The study was conducted in MDNP, Gabon (Chapter 1. Fig 1-1). The park covers an area of 5,028 km^2. The study area covers about 120 km^2 in the southeastern part of the park at an altitude of 50–800 m. The research station was located at 2° 20′ and 10° 34′ E. The vegetation is a complex mosaic of semi-primary forest, secondary forest, *Musanga cecropioides*-dominated forest, savanna, and swamp (Iwata and Ando 2007). This area typically experiences two seasons: the rainy season from mid-October to May and the dry season from June to September. Mean annual rainfall (2002–2006) was 1,777 mm (range: 1,583–2,163 mm). The mean monthly minimum and maximum

temperatures varied from 21.3°C to 24.1°C and 29.3°C to 33.7°C, respectively (Takenoshita *et al*. 2008).

4-2-2 Sampling of feces

The feces were collected in the forests of Boutiana and Douguetsi in MDNP in Gabon. In this national park, anthropological and ecological studies have been carried out since 2003, and a group of gorillas is now habituated (Ando *et al*. 2008). Chimpanzees are not yet well habituated, but they sometimes allow the approaching researchers to collect fresh feces. Elephants are one of most dangerous animals in this study area, but their numerous fresh feces are relatively easily collected. The fresh feces of western lowland gorillas were collected on November 23 and December 16, 2011, in Boutiana. From the volume and size of the feces, one fecal sample was judged to be from a male silverback gorilla (SBG) of this group, and the other the feces of an infant gorilla (IG). The feces of chimpanzees (CH1 and CH2) were collected on December 29, 2011, in the forest of Douguetsi adjacent to that of Boutiana. Fresh feces from an elephant (EP) was collected in Boutiana on December 20, 2011. A portion of feces free from contamination such as soil or dead leaves was sampled in an RNA later solution. Samples were stored in a dark place in the campsite of Boutiana until the end of the field research (January 8, 2012). Samples were then transported to the laboratories of the Research Institute of Tropical Ecology (IRET) at Libreville, where samples were placed in a refrigerator and later transported to Kyoto Prefectural University; there, samples were stored at -20°C until DNA extraction.

4-2-3 Culture-independent method

Bacteria were recovered from the fecal samples by centrifugation with exhaustive washing with phosphate-buffered saline to remove residual RNA later solution. The resultant bacterial pellets were subjected to DNA extraction with cell disruption by zirconia beads beating (Microsmash, TOMY, Tokyo) and a DNA stool mini kit (QIAGEN, Tokyo). After quantification by spectrophotometry, a portion (100 ng) of each DNA was subjected to PCR amplification of a partial 16S rRNA gene (V1-V2 region) using ExTaq polymerase (Takara, Kyoto). PCR primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 520R (5'-ACCGCGGCTGCGGC-3') (Lane 1991), were used to gain nearly 500 bp PCR amplicon in size. Both primers were attached with barcode sequences. The primers were supplied from Hokkaido System Science Co., Ltd. (Sapporo, Japan). PCR was performed under the following conditions: 3 min of initial denaturation at 95°C followed by 30 cycles (95°C for 30 s, 55°C for 40 s, and 72°C for 90 s) and final extension at 72°C for 4 min. The amplicons were purified by the PCR Clean-Up System (Promega, Madison, WI, USA).

4-2-4 Pyrosequence analysis

Pyrosequencing and sequence analysis was performed at Hokkaido System Science. Pyrosequencing was performed by the 454 Genome Sequencer FLX (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the manufacturer's instructions.

The sequences were cleaned by custom script on LINUX to remove sequences comprising a base-call other than A, T, C, or G. Then the sequences shorter than 250 bp were removed from whole sequence reads by the same application. The resultant cleaned sequence reads were further subjected to BLAST search by the stand-alone

program NCBI BLAST (http://www.ncbi.nlm.nih.gov/books/ NBK52640/) to remove sequences showing less than 50% alignment similarity with known sequences, because those sequences possibly contained chimeric sequences.

4-2-5 Taxonomic classification analysis on pyrosequencing data

The cleaned sequences from the previous step were assigned their phylotypes using DDBJ database according to QIIME 454 Tutorials (http://qiime.org/tutorials/tutorial.html) with 80% confidence threshold. In this taxonomic analysis, we defined sequences as identified OTUs (Operational Taxonomic Units) when their similarities to known sequences were lager than 90%. The sequences were defined as no-rank OTUs if their similarities to known sequences were smaller than 90%.

4-2-6 UniFrac cluster analysis

The cleaned sequences were compared in a pair-wise fashion by means of the UniFrac distance metric (Lozupone and Knight 2005). All steps were carried out in an automated fashion within QIIME (Caporaso *et al*. 2010). UniFrac analysis was carried out in a weighted fashion, which takes into account the relative proportions of each individual (Wu *et al*. 2010). Clustering was visualized for weighted UniFrac data using principal coordinate analysis (Gower 1966).

4-3 Results

4-3-1 Pyrosequencing

The total nucleotides of 16S rRNA genes detected from the feces, number of

sequence reads, and average read length are shown in Table 4-1. On average, pyrosequencing on fecal samples of IG, SBG, CH1, CH2, and EP yielded 16,898 reads with a read length of 390 nucleotides (10,860,585 nucleotides in total). Rarefaction curves calculated by Chao1 richness estimator in QIIME indicated that microbial diversity reached saturation at 14,000 sequence reads (Fig. 4-1).

4-3-2 Phylogenetic profile of the fecal bacteria at phylum and class levels

Firmicutes and Bacteroidetes were detected as major bacterial phyla in the feces of all animal studied. Then, Clostridia and Bacteroidia were detected as the major bacterial classes in the feces of all animal studied. Erysipelotrichi was detected as the major class of the fecal Firmicutes in chimpanzees (Fig. 4-2).

4-3-3 Phylogenetic profile of the fecal bacteria in gorillas at family and genus levels

The major bacterial families (>5% in total population) detected in the feces of IG were Prevotellaceae, Clostridiaceae, Ruminococcaceae, Eubacteriaceae, and Lachnospiraceae, each having 6,378 (37%), 1,764 (10%), 1,609 (9%), 818 (5%), and 786 (5%) sequences, respectively (Fig. 4-3A). These five families accounted for 66% of the total fecal bacteria in IG.

At the genus level, the fecal bacteria of IG was dominated by no-rank OTUs which relates to unknown Firmicutes (GU428814_1) followed by the OTUs assigned as *Prevotella*. Within no-rank OTUs at genus level, which covered 40% of the total sequence reads retrieved from the fecal bacteria of IG, the most prevalent no-rank OTU was suggested to relate with the sequence of unknown Firmicutes (GU428814_1), the second to unknown Firmicutes (AB262677_1), and the third to unknown Bacteroidetes

(AB547676_1).

In identified OTUs at genus level, which covered 60% of the total sequence read, the most prevalent OTU was similar to *Prevotella copri*, the second to *Prevotella oulorum*, and the third to *Clostridium indolis*. Their similarities with known sequences were from 92% to 96%.

For the fecal bacteria of SBG, Prevotellaceae, Lachnospiraceae, Clostridiaceae, and Veillonellaceae were the major families, each having 5,306 (33%), 3,062 (19%), 1,080 (7%), and 795 (5%) sequences, respectively (Fig. 4-3B). These four families accounted for 64% of the total fecal bacteria in SBG.

At the genus level, the fecal bacteria of SBG was dominated by no-rank OTUs which relates to unknown Bacteroidetes (EU728760_1) followed by the OTUs assigned as *Prevotella*. Within no-rank OTUs at genus level, which covered 34% of the total sequence reads retrieved from the fecal bacteria of SBG, the most prevalent no-rank OTU was suggested to relate with the sequence of unknown Bacteroidetes (EU728760_1), the second to unknown Firmicutes (GU429031_1), and the third to unknown Bacteroidetes (AJ009933_1). In identified OTUs at genus level, which covered 66% of the total sequence read, the most prevalent OTU was similar to *Oribacterium sinus*, the second to *Prevotella bryantii*, and the third to *Prevotella paludivivens*. Their similarities with known sequences ranged from 92% to 98%.

At the major families in the feces of gorillas, Ruminococcaceae and Eubacteriaceae presented in only the fecal bacteria of IG. These bacterial families presented in the fecal bacteria of SBG, however they were minor families. While Veillonellaceae were the major families in only the fecal bacteria of SBG. This babcterial family was not detected in the fecal bacteria of IG.

4-3-4 Phylogenetic profile of the fecal bacteria in chimpanzees at family and genus levels

The major families (>5% in total population) in the feces of CH1 were Lachnospiraceae, Prevotellaceae, Clostridiaceae, Oscillospiraceae, Erysipelotrichaceae, Ruminococcaceae, Veillonellaceae, and Eubacteriaceae, each having 2,420 (15%), 1,862 (11%), 1,317 (8%), 944 (6%), 935 (6%), 868 (6%), 815 (5%), and 734 (5%) sequences, respectively (Fig. 4-3C). These eight families accounted for 63% of the total fecal bacteria in CH1.

At the genus level, the fecal bacteria of CH1 was dominated by no-rank OTUs which relates to unknown Bacteroidetes (AB239491_1) followed by the OTUs assigned as *Prevotella*. Within no-rank OTUs at genus level, which covered 49% of the total sequence reads retrieved from the fecal bacteria of CH1, the most prevalent no-rank OTU was suggested to relate with the sequence of unknown Bacteroidetes (AB239491_1), the second to unknown Bacteroidetes (GQ131410_1), and the third to unknown Firmicutes (GU470893_1). In identified OTUs at genus level, which covered 51% of total sequence read, the most prevalent OTU was similar to *Oscillibacter valericigenes*, the second to *Prevotella oulorum*, and the third to *Prevotella oris*. Their similarities with known sequences ranged from 92% to 96%.

In the feces of CH2, the major bacterial families were Lachnospiraceae, Prevotellaceae, Erysipelotrichaceae, and Clostridiaceae, each having 3,505 (18%), 2,695 (14%), 2,533 (13%), and 1,886 (10%) sequences, respectively (Fig. 4-3D). These four families accounted for 55% of the total fecal bacteria in CH2.

At the genus level, the fecal bacteria of CH2 was dominated by no-rank OTUs which relates to unknown Firmicutes (GU470893_1) followed by the OTUs assigned as

Prevotella. Within no-rank OTUs at genus level, which covered 44% of the total sequence reads retrieved from the fecal bacteria of CH2, the most prevalent no-rank OUT was suggested to relate with the sequence of unknown Firmicutes (GU470893_1), the second to unknown Bacteroidetes (AB239491_1), and the third to unknown Bacteroidetes (GQ422745_1). In identified OTUs at genus level, which covered 56% of the total sequence read, the most prevalent OTU was similar to *Oribacterium sinus*, the second to *Prevotella oulorum*, and the third to *Prevotella oris*. Their similarities with known sequences were from 92% to 97%.

At the major families in the feces of chimpanzees, Oscillospiraceae Ruminococcaceae, Veillonellaceae, and Eubacteriaceae presented in only the fecal bacteria of CH1. These bacterial families presented in the fecal bacteria of CH2, however they were minor families.

4-3-5 Phylogenetic profile of the fecal bacteria in an elephant at family and genus levels

The major families (>5% in total population) in the feces of EP were Lachnospiraceae, Prevotellaceae, Clostridiaceae, and Eubacteriaceae, each having 1,357 (9%), 1,347 (9%), 1185 (8%), and 752 (5%) sequences, respectively (Fig. 4-3E). These four families accounted for 26% of the total fecal bacteria in EP.

At the genus level, the fecal bacteria of EP was dominated by no-rank OTUs which relates to unknown Firmicutes (EU281854_1) followed by the OTUs assigned as *Prevotella*. Within no-rank OTUs at genus level, which covered 52% of the total sequence reads retrieved from the fecal bacteria of EP, the most prevalent no-rank OTU was suggested to relate with the sequence of unknown Firmicutes (EU281854_1), the second to unknown Bacteridetes (AB501166_1), and the third to unknown Firmicutes

(AB596885_1). In identified OTUs at genus level, which covered 48% of the total sequence read, the most prevalent OTU was similar to *Prevotella ruminicola*, the second to *Prevotella copri*, and the third to *Prevotella oulorum*. Their similarities with known sequences ranged from 92% to 94%.

4-3-6 Common bacterial families and genus in the feces of gorillas, chimpanzees, and an elephant

At the family level, Prevotellaceae and Clostridiaceae were detected as major bacterial families in the feces of all animals. Excluding IG, Lachnospiraceae was detected as a major bacterial family in the feces of adult animals. Unidentified families accounted for >20% of the population in all animals (Fig. 4-3).

At the genus level, *Prevotella* was detected as the most dominant bacterial genus in the feces of all animals.

4-3-7 UniFrac cluster analysis

Weighted UniFrac analysis, which takes into account the information of abundance, shows the relative difference in composition of fecal microbiota of gorillas, chimpanzees, and an elephant (Fig. 4-4).

4-4 Discussion

Intestinal microbiota play an important role in digestion, absorption of nutrients, and the host's health (Sekirov *et al*. 2010, Kau *et al*. 2011). This theory is substantiated by many works in humans and their model experimental animals, as well as in some livestock. Such a relationship is now understood as a context of co-evolution

(Amato 2014). Studies on the intestinal microbiota of wild animals are important for recognizing the co-evolution of the host and its intestinal microbiota. However, the intestinal microbiota in wild animals have not been well studied due to the technical difficulties of cultivation in field conditions.

In developing culture-independent analyses, some of the studies succeeded in showing the characteristics of the intestinal microbiota of wild animals (Uenishi *et al*. 2007, Glad *et al*. 2010). However, the techniques allowed analysis only of the top 20 to top 200 levels of bacteria. The recent development of the so-called Omics approach allows for analysis of some 1,000 bacterial OTUs. This development is helpful for better understanding the characteristics of intestinal microbiota of particular wild animals.

In the present study, we believe that the host-specific characteristics of the fecal microbiota of wild western lowland gorillas, central chimpanzees, and a forest elephant that are living in a relatively narrow area and foraging quite similar food resources are shown. In previous studies, by using metagenomic analyses, Bacteroidetes and Firmicutes were shown to be the major intestinal bacterial phyla of various mammals, including gorillas, chimpanzees, and elephants in the wild and in captivity (Ley *et al*. 2008, Moller *et al*. 2013). Our results also demonstrate that the Firmicutes and Bacteroidetes were the common intestinal bacteria for gorillas, chimpanzees, and forest elephants at the phylum level. However, the relative proportion of these phyla were not the same for host animal species tested (Fig 4-2). Therefore, as shown by UniFrac analysis, the fecal microbiota in gorillas and in chimpanzees were different, and that of an elephant was further distant from those of gorillas and chimpanzees.

Among the identified OTUs at genus levels, excluding SBG, *Prevotella*

oulorum was detected as the dominant OTU of all animals, and *Prevotella oris* was the dominant OTU of chimpanzees. In addition to these, *Prevotella copri* was detected as the dominant OTU of IG and EP. However, only a few identified OTUs were commonly shared by three different animal species.

The most important finding of this study is the quantitative importance of no-rank OTUs in the fecal microbiota of all animals tested. In our results at genus level, no-rank OTUs covered from 35% (SBG) to 52% (EP) of the total sequences in each animal subject. Moreover, they constituted the richest OTUs in all animals. As indicated above, the similarity of sequences of no-rank OTUs to those registered in the data bank was lower than 90% in this study. These no-rank OTUs are, accordingly, unknown bacteria that have never been isolated or characterized. This seems to be in contrast to those analyzed for human fecal microbiota. In the latter case, no-rank OTUs constituted less than 20% of the total OTUs detected when analyzed by the same method (Inoue, personal communication). Human-associated bacteria have been studied more intensively than those of wild animals for a long time. The database for human-associated bacteria is obviously better documented than that for wild animals. To some extent, the rumen bacteria of ruminant livestock have been focused so far due to their economic importance (Flint *et al*. 2008, Duan *et al*. 2009, Jami and Mizrahi 2012). This may limit the application of metagenomic analyses on fecal microbiota in wild animals particularly mono-gastric animals. Because of this limitation, only the phylum-level comparison has been reported so far, which only allows for the rough comparison between host animal species and may not allow for characterization in detail. As shown in the recent reports based on metagenomic analyses (Ley *et al*. 2008, Bhatt *et al*. 2013), phylum-level analyses can reveal that the mammals possess nearly

the same composition of microbiota. For example, in our study, an elephant has a similar composition to those of gorillas and chimpanzees at the phylum level (Fig. 4-2). However, its composition was clearly different from those of gorillas and chimpanzees when a comparison was made with family-level analyses (Fig. 4-3). And the level of diversity in intestinal microbiota of elephant was far bigger than great apes (Fig. 4-1). The difference was, in fact, attributable mostly to no-rank OTUs. We believe that no-rank OTUs should be analyzed in detail for the better understanding and comparison of intestinal microbiota of animals except for humans. To understand the co-evolution between the host and its intestinal microbiota, further efforts to isolate and identify the unknown bacteria corresponding to no-rank OTUs are of importance.

Table 4-1. Pyrosequencing results

	IG	PG	CH 1	CH 2	EP
Total 16S rRNA gene (nucleotides)	10,065,107	10,121,642	9,684,893	12,706,879	11,724,402
Number of sequence reads	17,437	16,300	15,736	19,503	15,514
Average read length (nucleotides)	414.22	384.81	386.16	386.03	378.87

*Adapted and modified from Table 1 in Tsuchida *et al*.2014d.

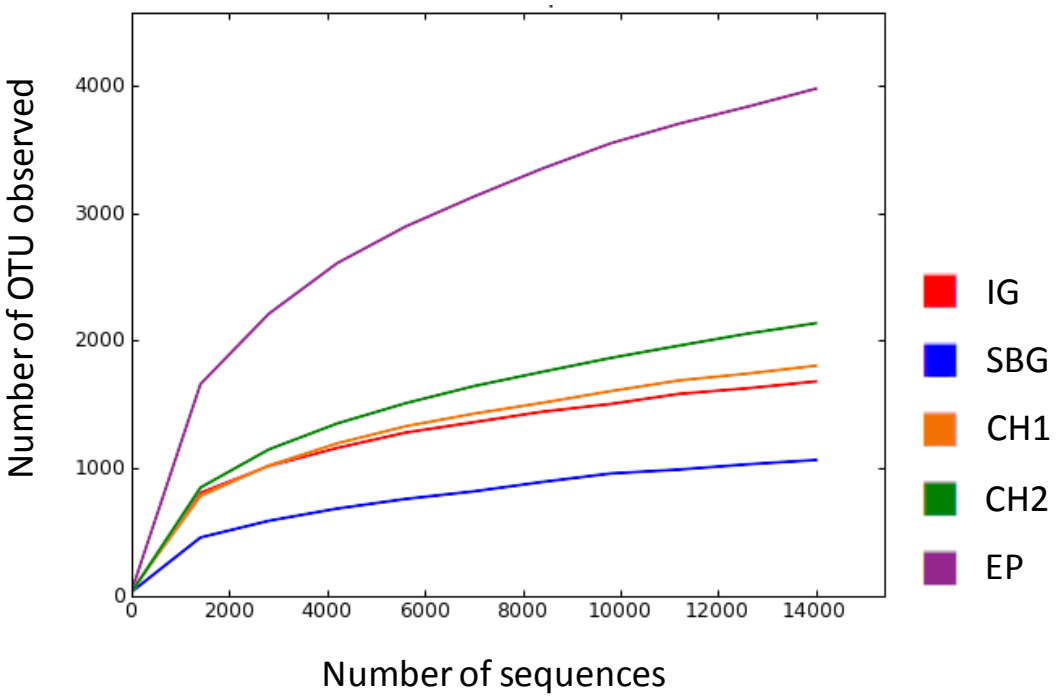


Fig. 4-1. Rarefaction curves of the number of sequence reads of each individual in this study

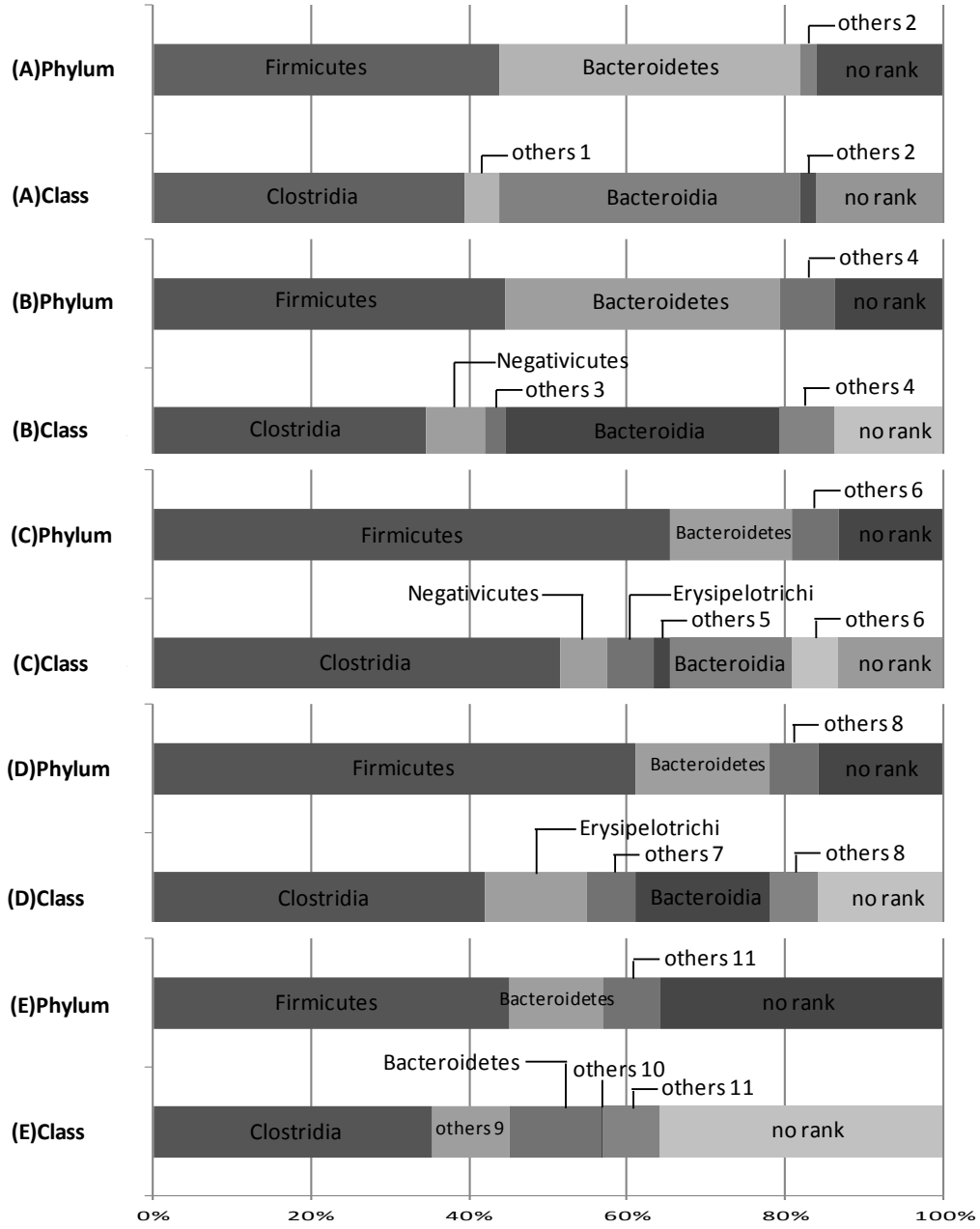


Fig. 4-2. Phylogenetic profile of the fecal bacteria at phylum and class levels of (A) IG, (B) SBG, (C) CH1, (D) CH2, and (E) EP. Others1, 3, 5, 7, and 9: Other minors belong to Firmicutes. Others 10: Other minors belong to Bacteroidetes. Others were the phylum and class whose percentages accounted for <5%. No rank indicates the sequences unidentified by BLAST search.

*Adapted and modified from Fig 1 in Tsuchida *et al*.2014d.

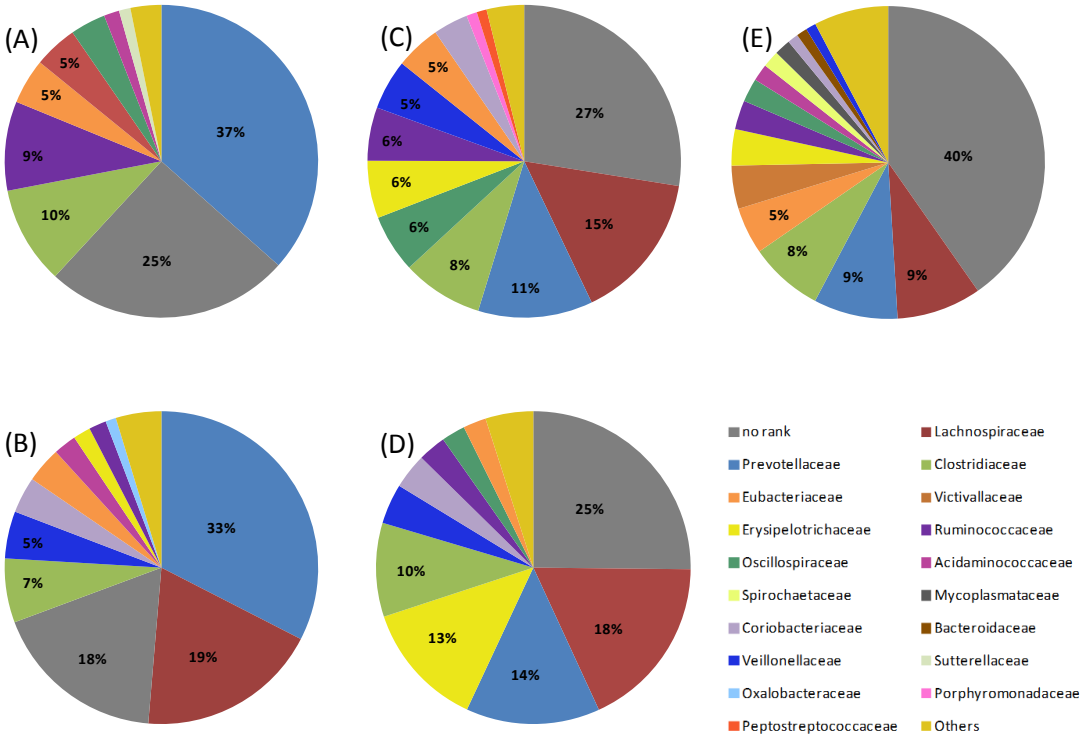


Fig. 4-3. Phylogenetic profile of the fecal bacteria at family level of (A) IG, (B) SBG, (C) CH1, (D) CH2, and (E) EP. Others were the families whose percentages accounted for <1%. No rank indicates the sequences unidentified by BLAST search.

*Adapted and modified from Fig 2 in Tsuchida *et al*.2014d.

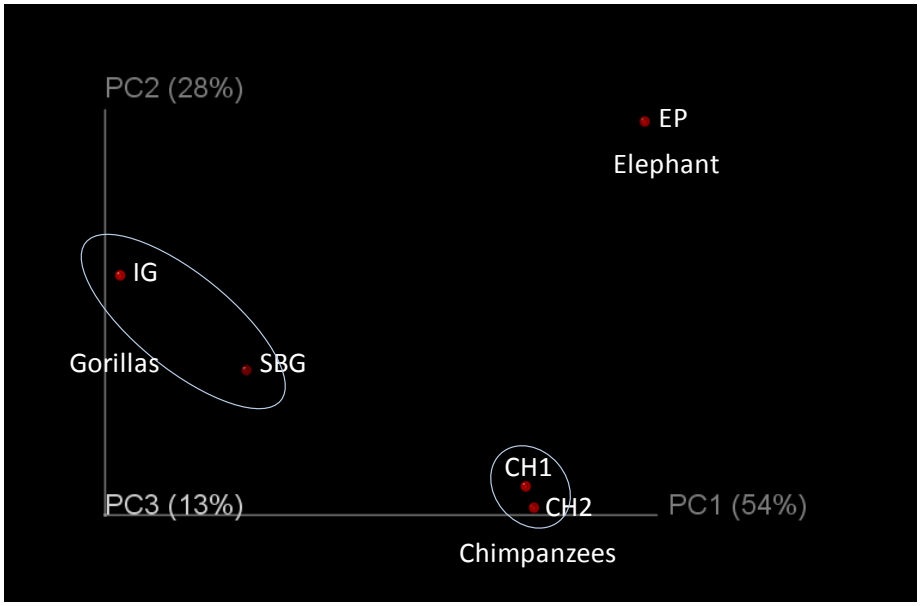


Fig. 4-4. Comparison of the relative abundance of the fecal microbiota in each individual using weighted UniFrac.

*Adapted and modified from Fig 3 in Tsuchida *et al*.2014d.

Chapter 5

General discussion and Conclusion

5-1 General discussion

In this study, the author discovered one novel species of bifidobacteria and one novel species of lactobacilli in western lowland gorillas using culture-dependent methods.

In chapter 2, *Bifidobacterium moukalabense* sp. nov., which was isolated from wild western lowland gorillas, central chimpanzees, and a forest elephant in MDNP, is described. Gorillas, chimpanzees, and forest elephants in MDNP are sympatric, depending on nearly the same food varieties. *B. moukalabense* were isolated from all gorilla feces during the study (from 2009 to 2013). These bifidobacteria are distinctive on the BS plate, producing orange-colored colonies. Although this bifidobacterium was isolated from the feces of chimpanzees and elephants, not all feces of these animals carried these particular bifidobacteria. Therefore, it is suggested that *B. moukalabense* was a gorilla-specific autochthonous intestinal bacteria in MDNP. *B. moukalabense* belongs to *B. adolescentis*-phylogenetic group, which includes *B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. dentium*, and *B. angulatum* as members (Sgorbai and London 1982). Three species—*B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*—in this group are detected in human feces with a high frequency; therefore, these three species are considered autochthonous intestinal bacteria of humans (Matsuki *et al*. 1999, Turroni *et al.* 2009, Mitsoka 1990). In previous studies, substantial amylolytic activity was detected in these three bifidobacteria (Crittenden *et al.* 2001). *B. angulatum* and *B. dentium* were occasionally detected in humans

(Matsuki *et al*. 1999, Lamendella *et al*. 2008), and several strains of *B. angulatum* were isolated from wild chimpanzees in Bossou, Guinea (Ushida *et al*. 2010). *B. moukalabense*, as a novel species belonging to *B. adolescentis*-group, is mostly associated with wild western lowland gorillas. According to the host range of these six species of bifidobacteria, it is suggested that the speciation of bifidobacteria, particularly those belonging to the *B. adolescentis* group, occurred within the human GIT. *B. moukalabense* is actually carried by wild gorillas, with occasional horizontal transmission to sympatric members in the African rain forest. As mentioned above, chimpanzees and elephants seem not to be specific hosts of *B. moukalabense*, due to its relatively low frequency. Chimpanzees, rather, carry more human-associated bifidobacteria, and elephants are not true hosts for the bifidobacteria. Gorillas are frugivores/folivores with the occasional ingestion of insects (Tutin and Fernandez 1993, Yamagiwa and Basabose 2006). They have apparently kept the eating habits of the common ancestor of humans and gorillas. Chimpanzees also rely on fruits and leaves, but they eat substantial amounts of animal meat (Mitani and Watts 2001, Gilby 2006). It is noteworthy that early humans, *Homo habilis* and *H. erectus*, were hunter-gatherers who relied substantially on animal meat (McHenry and Coffing 2000, *Leonard et al.* 2003). Our ancestors became omnivores. After agriculture began some 12,000 years ago, our ancestors started increasing their consumption of starch-rich foods (Salamini *et al.* 2002). Bifidobacteria in the human GIT may have speciated (or diversificated) tremendously in adapting to such tremendous changes in food habits (from frugivores/folivores to omnivores) and digestive system (teeth, in particular) of the hosts. At present, we are analyzing the genomic structure of several strains of *B. moukalabense* (Tsuchida *et al*. 2014b). Our preliminary results indicate that the genome

of this species is larger (ca. 2.5 Mb) than that of human-associated bifidobacteria (< 2.0 Mb). The original genes that differentiate *B. moukalabense* from other human-associated bifidobacteria are mostly hypothetical proteins or unknown, although some particular functionalities were assigned by KEGG and COG, such as anthranilate phosphoribosyltransferase, dihydroorotate dehydrogenase, hydantoin racemase, and hydantoinase.

As shown in chapter 3, the author succeeded in isolating and identifying *Lactobacillus gorillae* sp. nov. from the feces of captive and wild western lowland gorillas. This novel species of lactobacilli is, therefore, considered to be an autochthonous intestinal bacterium of western lowland gorillas.

L. gorillae belongs to the *L. reuteri*-phylogenetic group. *L. reuteri* are repeatedly isolated from carnivores/omnivores, including humans; it is considered that *L. reuteri* are allochthonous intestinal bacteria in carnivore/omnivore animals. Herbivores are not the major host of this group of lactobacilli (Endo *et al.* 2010). Within this group, *L. fermentum*, the closest neighbor of *L. gorillae*, was isolated from humans with a high frequency; however, this species was rarely isolated from other animals. *L. fermentum* is, therefore, considered to be an autochthonous intestinal bacterium in humans. As discussed above, *B. moukalabense* is categorized in the human-associated *B. adolescentis* group, and its closest neighbor, *B. dentium*, is a typical human-type bifidobacterium. *L. gorillae* is categorized in the *L. reuteri* group, whose host range is wider than that of the *B. adolescentis* group. However, the closest neighbor of *L. gorillae* is *L. fermentum*, a typical human-associated lactobacillus. Considering the phylogenetic relationship between gorillas and humans, an analogy is suggested between the host relationship (gorilla vs human) and the relationship between

autochthonous lactic acid bacteria (*B. moukalabense* vs *B. dentium*, *L. gorillae* vs *L. fermentum*). Since *L. reuteri* has a wide range of hosts, this species is considered rather universal, not having a specific host among carnivores and omnivores (Mitsuoka 1992). However, the presence of host-specific strains of *L. reuteri* has been demonstrated (Casas and Dobrogosz 1997). It is plausible that the speciation of *L. reuteri* occurred to adapt to its wide range of animal hosts. This may be true for *L. gorillae.* According to phylogenetic, phenotypic, and physiological analyses in chapter 3, 16S rRNA gene sequences and *pheS* gene sequences of strains of *L. gorillae* were almost identical. Interestingly, ribotyping analyses showed that one strain from a captive adult male gorilla (KZ03) has the exact same ribotype pattern as does strain GG02 from a wild individual. Furthermore, this captive gorilla harbored the strain KZ02 that showed the same ribotype pattern as did strain KZ01^{T}, isolated from a captive adult female gorilla. From phenotypic analyses, GG02 and KZ03 both showed similar sugar utilization patterns, while KZ01^{T} and KZ02 showed sugar utilization patterns similar to that of *L. fermentum.* GG02 and KZ03 could degrade D-xylose, arbutin, cellobiose, and trehalose, but KZ01^{T} and KZ02 could not degrade these substrates. Considering the food in the wild, utilization of D-xylose, cellobiose, and arbutin is of importance because D-xylose and cellobiose constitute hemicelluloses and cellulose of the cell walls of plants, and arbutin is phenol glycoside that has cytotoxicity and antibacterial activity and is contained in wild fruit and leaves such as *Pyrus* spp. (Kundaković *et al.* 2014). The natural diet for gorillas in the wild should be more fibrous and richer in anti-nutritional compounds such as phenol glycoside than food offered in conditions of captivity. The ability of wild ribotypes, represented by GG02 and KZ03, to degrade polymer constituents and plant toxin suggests the adaptation of these strains to wild feeding

conditions more than other ribotypes, represented by KZ01^T and KZ02. There was a difference in NaCl tolerance between strains from captive individuals (KZ01^T, KZ02, and KZ03) and wild individuals (GG02). The former showed substantially higher tolerance to NaCl, which may be induced by regular food in zoos that contains sufficient amounts of minerals, while in wild conditions, Na is often depleted from gorillas' food, leading gorillas to selectively ingest unusual foods such as decayed wood (Rothman *et al.* 2006, Yamagiwa *et al.* 2005). It is suggested that the phenotypes and ribotypes of the strains have changed with the change in the food consumed. This may be a case of speciation of the species, but further elucidation at the level of genome on these *L. gorillae* isolates seems to be indispensable.

In chapter 4, the author characterized the intestinal microbiota of gorillas, chimpanzees, and elephants living sympatric in MDNP, using culture-independent methods. In previous studies, using metagenomic analysis, Bacteroidetes and Firmicutes were shown to be the major intestinal bacterial phyla of various mammals, including gorillas, chimpanzees, and elephants in the wild and in captivity (Ley *et al*. 2008, Moller *et al*. 2013). The results of this study also demonstrate that Firmicutes and Bacteroidetes were the most common intestinal bacteria for gorillas, chimpanzees, and forest elephants at the phylum level. At the class level, Clostridia and Bacteroidia were common intestinal bacteria for all animals studied, a result that is almost identical to those shown in previous studies (Ley *et al*. 2008, Moller *et al*. 2013, Dougal *et al.* 2013, Caporaso *et al.* 2011). In the case of chimpanzees, however, Erysipelotrichi was also detected as the major class of their intestinal microbiota. In the literature, Erysipelotrichi was recognized common member of various intestinal microbiota (Handl *et al*. 2011, Zhang and Chen 2010, Peris-Bondia *et al.* 2011). It is noteworthy that this particular

class increased in response to a high fat diet in mice (Greiner *et al*. 2011). Chimpanzees consume more animal proteins as compared with gorillas and forest elephants. Such an eating habit may affect the development of bacteria belonging to the Erysipelotrichi class.

One of the most striking observations about this NGS-based metagenome is the higher diversity of elephant microbiota as compared to those of chimpanzees and gorillas. However, the composition of intestinal microbiota of a forest elephant is far more ambiguous than those of chimpanzees and gorillas; no-rank OTUs, even at the phylum level, covered from 14% (SBG) to 36% (EP) of the total sequences obtained in each animal, constituting the richest OTUs of all animals. In this study, no-rank OTU means the presence of unknown bacteria even at the phylum level in these wild animals. Gorillas and chimpanzees are primates and our evolutionary neighbors. Perhaps, they share the bacteria first detected in human feces at a substantial level. Accordingly, the majority of sequences in gorillas and chimpanzees raised by this metagenomic analyses can be identified with databases in which human intestinal bacteria have been deposited more intensively than those from other sources. However, no-rank OTUs still constitute the largest group at the family level for all animals. This may be caused by insufficient isolation work for the intestinal bacteria in wild animals.

5-2 Conclusion

The present study demonstrates the presence of specific intestinal microbiota of western lowland gorillas using culture-dependent methods and culture-independent methods. A novel species, *B. moukalabense* sp. nov., can be considered to be gorilla-specific autochthonous intestinal bacteria with an occasional horizontal transfer

from gorillas to other sympatric animals in MDNP. Since this species has a close phylogenetic relationship with the human-type bifidobacterium *B. dentium*, genomic comparison between *B. moukalabense* and *B. dentium* (or *B. catenulatum* or *B. pseudocatenulatum*) would make clear the adaptation of bifidobacteria to the human GIT and the reasons for vast speciation of bifidobacteria in humans.

A novel species, *L. gorillae* sp. nov., was a gorilla-specific autochthonous intestinal bacterium. This bacterium also has a close phylogenetic relationship with the human-type lactobacillus, *L. fermentum*. Again the genomic comparison between *L. gorillae* and *L. fermentum* would make clear the adaptation of lactobacillus to the human GIT.

The culture-independent metagenome showed the host-specific intestinal microbiota, though these species are living in a relatively narrow area and foraging for quite similar food resources. Unfortunately, bifidobacteria were technically omitted by the present approach using amplification of the V1–V3 region of the 16S rRNA gene. Further precise study should be conducted to match a culture-dependent approach and a culture-independent approach toward wild animals.

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Summary

Chapter 1

Intestinal microbiota of the mammal develop a very complex ecosystem with vast diversity after birth, which show large differences among animal species and individuals. Such an ecosystem may evolve in a host-specific manner. The host-specific development of intestinal microbiota is probably caused by the host's digestive system and food habits. Especially, some in bifidobacteria and lactobacilli species should be regarded as allochthonous intestinal bacteria, although it is still difficult to reveal how and what kind of selection was applied to the intestinal bacteria for the establishment of such host-specific intestinal microbiota.

In this chapter, the author explained the aims of this study. The first aim was to investigate lactic acid bacteria in non-human wild primates to characterize host-specific lactic acid bacteria in wild and captive western lowland gorillas in Moukalaba-Doudou National Park (MDNP), Gabon, because further comparisons of gorilla-specific lactic acid bacteria and those in humans may reveal the diversification and selection of lactic acid bacteria in the human GIT.

The second aim of this study was to evaluate environmental effects on intestinal microbiota. In this respect, the author intended to study the intestinal microbiota of western lowland gorillas, central chimpanzees, and a forest elephant in the wild using pyrosequencing analyses because gorillas and chimpanzees at our study site are sympatric, depending on nearly the same food varieties.

Chapter 2

Gram-staining positive anaerobic rods were isolated from the feces of a wild lowland gorilla, central chimpanzees, and a forest elephant in MDNP, and these isolates were taxonomically investigated. Based on phylogenetic analyses and specific phenotypic characteristics, these strains belonged to the genus *Bifidobacterium.* Phylogenetic analysis of its 16S rRNA gene sequence revealed that these isolates form a single monophyletic cluster. The *hsp60* sequence also supports these relationships. *Bifidobacterium moukalabense* sp. nov. was proposed. This novel species was considered to be a major *Bifidobacterium* in the intestines of wild lowland gorillas, chimpanzees, and elephants in MDNP.

Chapter 3

Gram-staining positive anaerobic rods were isolated from the feces of captive and wild western lowland gorillas. These strains were taxonomically investigated. Phylogenetic analysis based on 16S rRNA gene sequences and specific phenotypic characteristics demonstrated that these strains belong to the genus *Lactobacillus reuteri* phylogenetic group. Phylogenetic analysis of their 16S rRNA gene sequences revealed that these isolates form a single monophyletic cluster. The *pheS* sequences also support these relationships. Therefore, based on phylogenetic, phenotypic, and physiological evidence, these strains represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus gorillae* sp. nov. was proposed. This novel species is considered to be a major *Lactobacillus* in the intestines of western lowland gorillas.

Chapter 4

Intestinal microbiota play an important role in digestion and the host's health.

Furthermore, their composition is complex, with large differences between animal species. Intestinal microbiota have been intensively studied in humans, whereas those in animals, especially in the wild, have not been thoroughly studied. In this chapter, I focused on the intestinal microbiota of wild western lowland gorillas, chimpanzees, and an elephant in MDNP by using pyrosequencing analysis for understanding their characteristics. In almost all animal feces, Prevotellaceae, Clostridiaceae, and Lachnospiraceae were detected as major bacterial families. At the genus level, no-rank Operational Taxonomic Units (OTUs), 80%–90% identities with known sequences, covered a major fecal microbiota, which seemingly determined the enterotype of the host. However, in principal coordinate analysis using weighted UniFrac, their fecal bacteria were clustered by the species of the host. The result of the present study suggests that it is necessary for no-rank OTUs and minor populations of fecal bacteria to be analyzed in detail to understand the true characteristics, such as functionality, of intestinal microbiota.

Chapter 5

A gorilla-specific *B. moukalabense* belongs to the *B. adolescentis*-phylogenetic group that includes mostly human-associated bifidobacteria. The scientific value of this novel species was discussed in this chapter. Genomic comparison between *B. moukalabense* and the *B. adolescentis*-phylogenetic group would clarify the adaptation of bifidobacteria to the human GIT and the reasons for the vast speciation of bifidobacteria in humans. Gorilla-specific *L. gorillae* belongs to the *L. acidophilus*-phylogenetic group that includes mostly human-associated lactobacilli. Our isolates showed the small difference; one strain from a captive gorilla (KZ03) has the

completely same ribotyping pattern as strain GG02 from a wild individual, and these strains degrade polymer constituents and plant toxins. It is suggested that the phenotypes and ribotypes of the strains have been changed with the change in diet. This may be a case of speciation of the species, but further elucidation at the genome level of these *L. gorillae* isolates seems to be indispensable.

The common intestinal bacteria for gorillas, chimpanzees, and forest elephants at various classification levels were discussed in this chapter. The composition of intestinal microbiota of a forest elephant was discussed. Its composition was far more ambiguous than those of chimpanzees and gorillas; no-rank OTUs, even at the phylum level, covered from 14% (SBG) to 36% (EP) of the total sequences obtained in each animal, constituting the richest OTUs in all animals. No-rank OTUs in this study mean the presence of unknown bacteria, even at the phylum level, in these wild animals.

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