Microbial community structure analysis in *Acer palmatum* bark and isolation of novel bacteria IAD-21 of the phylum *Abditibacteriota* (former candidate division FBP)

Kazuki Kobayashi[1] and Hideki Aoyagi[1,2]

[1] Division of Life Sciences and Bioengineering, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan
[2] Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

Corresponding author
Hideki Aoyagi,
aoyagi.hideki.ge@u.tsukuba.ac.jp

ABSTRACT

Background: The potential of unidentified microorganisms for academic and other applications is limitless. Plants have diverse microbial communities associated with their biomes. However, few studies have focused on the microbial community structure relevant to tree bark.

Methods: In this report, the microbial community structure of bark from the broad-leaved tree *Acer palmatum* was analyzed. Both a culture-independent approach using polymerase chain reaction (PCR) amplification and next generation sequencing, and bacterial isolation and sequence-based identification methods were used to explore the bark sample as a source of previously uncultured microorganisms. Molecular phylogenetic analyses based on PCR-amplified 16S rDNA sequences were performed.

Results: At the phylum level, *Proteobacteria* and *Bacteroidetes* were relatively abundant in the *A. palmatum* bark. In addition, microorganisms from the phyla *Acidobacteria*, *Gemmatimonadetes*, *Verrucomicrobia*, *Armatimonadetes*, and *Abditibacteriota*, which contain many uncultured microbial species, existed in the *A. palmatum* bark. Of the 30 genera present at relatively high abundance in the bark, some genera belonging to the phyla mentioned were detected. A total of 70 isolates could be isolated and cultured using the low-nutrient agar media DR2A and PE03. Strains belonging to the phylum *Actinobacteria* were isolated most frequently. In addition, the newly identified bacterial strain IAP-33, presumed to belong to *Acidobacteria*, was isolated on PE03 medium. Of the isolated bacteria, 44 strains demonstrated less than 97% 16S rDNA sequence-similarity with type strains. Molecular phylogenetic analysis of IAD-21 suggested it belongs to *Abditibacteriota*. Culture of the strain IAD-21 was deposited in Japan Collection of Microorganisms (JCM) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as JCM 32665 and DSM 108248, respectively.

Discussion: Our results suggest that a variety of uncultured microorganisms exist in *A. palmatum* bark. Microorganisms acquirable from the bark may prove valuable for academic pursuits, such as studying microbial ecology, and the bark might be a promising source of uncultured bacterial isolates.

INTRODUCTION

The total number of microorganisms existing on the earth is speculated to range from 10^{29} to 10^{30} organisms (*Whitman, Coleman & Wiebe, 1998*; *Kallmeyer et al., 2012*). It is reported that the number of operational taxonomic units (OTUs) detected from one g of soil is up to 52,000 (*Roesch et al., 2007*). The number of bacterial species that currently have been isolated, investigated with regards to physiological properties, and assigned scientific names is about 15,000 (*Parte, 2018*). This is only 1% of the total number of bacterial species presumed to exist on earth, and the remaining 99% of uncultured microorganisms is called the "microbial dark matter" (*Ledford, 2015*). Until now, only cultivable microorganisms among the 1% have been used to comprehend the overall microbial ecosystem and identify novel useful genes, but the exploration of these cultivable microbes have reached a plateau in recent years (*Puspita et al., 2012*). Since the microbial dark matter is expected to potentially impact the current status of academic and industrial fields, comprehensive environmental genome analyses are being conducted around the world (*Rinke et al., 2013*; *McCalley et al., 2014*). However, unraveling microbial functions, which cannot be elucidated from the nucleotide sequence alone, or the practical utilization of uncultured microorganisms, requires pure culture isolation and cultivation (*Stewart, 2012*). Since cultivation of the remaining 99% of microorganisms holds great potential, exploration and isolation of microorganisms from various environments are desirable.

Numerous analyses on symbiotic microorganisms have been conducted for many terrestrial plants, and their microbial community structures are determined not only by plant species, but also by factors such as plant organs and environmental factors (*Wieland, Neumann & Backhaus, 2001*; *Schlaeppi et al., 2010*; *Edwards et al., 2015*; *Zarraonaindia et al., 2015*). Many of these microorganisms provide benefits to plants, such as the promotion of plant growth (*Köberl et al., 2013*), modification of plant-hormone production (*Bodenhausen et al., 2014*), and resistance to disease (*Berendsen, Pieterse & Bakker, 2012*). To understand the plant-microbial symbiotic relationship and its impact on the ecosystem, comprehensive analysis of the plant symbiotic microbial community structure and further isolation of symbiotic microorganisms, including uncultured microorganisms, are necessary. For example, in the case of agricultural crops and model plants, including *Arabidopsis thaliana*, exhaustive analyses of symbiotic microorganisms' function and community structure have been performed using both culture-independent and culture-dependent methods (*Delmotte et al., 2009*; *Manter et al., 2010*; *Vorholt, 2012*; *Comby et al., 2016*). However, for some plant types, the exploration of microbial resources has not been sufficiently conducted yet. *Tanaka et al. (2012)* focused on the rhizosphere of aquatic plants, which have not been thoroughly investigated for studying symbiotic microbial communities, and isolated *Armatimonas rosea* YO-36^T (Former Candidate division OP10) from the roots of *Phragmites australis*. *Tanaka et al. (2017)* also investigated the roots of the aquatic plants *Iris pseudacorus* and *Scirpus*

juncoides, and isolated microorganisms belonging to *Acidobacteria* and *Verrucomicrobia*, which are relatively difficult to cultivate. Microorganisms isolated from such aquatic plants are relatively novel, even if they belong to taxa with high cultivation frequency. Since environmental samples that have not been explored thus far lack information on microbial communities and isolates in databases, it is suggested that the novelty of cultured microorganisms is necessarily high from such unexplored potential microbial resources.

Analyses of the structures of microbial communities present on trees have been previously conducted (*Mocali et al., 2003*; *Moore et al., 2006*; *Taghavi et al., 2009*; *Redford et al., 2010*; *Filteau et al., 2010*; *Laforest-Lapointe, Messier & Kembel, 2016a*, *2016b*). However, very few studies have focused on the tree bark. The bark refers to the outer side of the cambium surrounding the xylem of the tree and is composed of an inner bark, which is the living tissue consisting of phloem, and an outer bark, which is the dead tissue of the cork. The bark is composed of polysaccharides (cellulose, hemicellulose), pectin substances, phenolic polymers such as lignin and high molecular weight tannins, and cross-linked polyesters such as suberin and cutin. The bark contains greater amounts of extracts (polyphenol and suberin), minerals, and lignin than the center of the tree (*Feng et al., 2013*). As a protective tissue, the bark consists of compounds that are resistant to microbial degradation, such as suberin (*Baldrian, 2017*). In addition, the bark is impregnated with resin that inhibits the growth of microorganisms (*Baldrian, 2017*). The bark protects the cambium from precipitation, heat, frost, and UV radiation and acts as a barrier against the attack of bacteria, fungi, parasitic plants, insects, and animals (*Sakai, 2001*). By adapting to tree bark, microorganisms may be able to acquire a stable habitat. In the case of bark (especially old bark), the tree canopy blocks precipitation and UV irradiation, and there is less disturbance than in other tissues such as leaves and branches, suggesting that microorganisms can stably inhabit areas for a long time (*Leff et al., 2015*). Further, microorganisms can colonize microsites such as cracks and lenticels, which represent a more favorable environment for microbial growth because they retain humidity and nutrients (*Buck, Lachance & Traquair, 1998*), and the symbiotic microorganisms can utilize plant biomass and photosynthetic products as carbon sources in such a stable habitat. Therefore, the bark presents a suitable habitat for slow-growing microbes and those susceptible to disturbance. However, compared with other tissues such as leaves and rhizosphere, microbial community structure analysis, and isolation of microorganisms (especially bacteria) including uncultured microorganisms from the bark have not been sufficiently performed.

Shen & Fulthorpe (2015) revealed the differences among the microbial community structures within the tree branches of the species *Acer negundo*, *Ulmus pumila*, and *U. parvifolia*, using isolation of the microorganisms and various culture-independent analyses. *Ulrich, Ulrich & Ewald (2007)* demonstrated the impact of different hybrid poplar clones on the endophytic community structure in branches and leaves using terminal restriction fragment length polymorphism analysis and analyzed the microbial community structure within the branches and leaves of poplar trees using isolation of the microorganisms and clone analysis. However, these two studies used tree branches as the source material, where the environment is completely different from that of the bark.

In addition, *Aschenbrenner et al. (2017)* revealed the microbial community structure of *Acer pseudoplatanus* bark, symbiotic moss, and lichens using next generation sequencing. Interestingly, there are a few studies that suggested the bark microbial community is different from that in other organs. *Martins et al. (2013)* investigated the cultivation and isolation of microorganisms from grapevine and reported that bacterial genera obtained from the bark differed from those obtained from the fruits and leaves. *Leff et al. (2015)* conducted a culture-independent analysis of *Ginkgo biloba* bark, branches, young branches, and leaves using high throughput 454 pyrosequencing and showed that the diversity of microbial communities in the old bark was the highest. In addition, phyla containing bacterial species that are generally difficult to culture, or are uncultured, such as *Acidobacteria*, *Armatimonadetes*, and the candidate division WYO (*Serkebaeva et al., 2013*; *Weiss et al., 2015*), were detected in higher proportions in the bark than in other tree organs. However, since there are few reported cases of analysis of the microbial community structure of bark samples and the isolation (acquisition) of uncultured microorganisms at higher taxonomic levels such as the phylum or class level, there is insufficient evidence to deduce whether bark is an excellent source of uncultured microorganisms.

Nonetheless, previous observations suggested that tree bark may harbor special microbial communities and that isolation and analysis of microorganisms from bark may provide insights into unknown microbial ecosystems and tree-microbial symbiosis. In the current study, we targeted the bark of *Acer palmatum*, which is a deciduous broad-leaved tree widely growing in Japan, and analyzed the microbial community structure using MiSeq-based next generation sequencing. In addition, we attempted to isolate and cultivate microorganisms by standard methods using low-nutrient agar media.

MATERIALS AND METHODS

Sample collection and pre-treatment

Bark sample was collected from mature *Acer palmatum* from the Ichimura Foundation for New Technology Botanical Research Gardens, Atami, Shizuoka, Japan (35.107336 N, 139.047729 E) using sterile tweezers and scissors. *Acer palmatum* trees used in this study were at least 80 years old or more and naturally occurring. The botanical research garden is 277–310 m above sea level, along a gentle slope facing south-south-east. Although the botanical garden is an artificially landscaped Japanese garden with artificially planted plants, it also contains several natural plants. A sample for culture-independent analysis was collected in November 2015, and a bark sample for microbial isolation was collected in June 2016. Bark samples for both analyses were collected from the same position (at a height of 1.0–1.5 m) on the same single tree. Further, in order to re-analyze the microbial community structure by culture-independent analysis, we collected three samples (at heights of 1.0, 1.5, and 2.0 m) from each of two individual trees (one being the previously analyzed tree) in February 2018. Approximately 4.7 g of bark fragments from the surface to a depth of about two mm were collected. Since the thickness of the bark (phloem and periderm) of another member of the same genus, *Acer rubrum*, is 0.8 ± 0.03 mm (*Hammond et al., 2015*), it was considered that the bark area should be covered by this sampling and that the collected sample contains both epiphyte and

endophyte in this range. The collected bark samples were minced using sterilized tweezers and scissors, suspended in 40 ml of phosphate-buffered saline, thoroughly mixed by vortexing, and sonicated at 42 kHz for 3 min using a Bransonic Ultrasonic Cleaner 3510J-DTH (Branson Ultrasonic Corporation, Danbury, CT, USA) to detach the microorganisms adhering to the bark surface. The microbial suspensions from the bark samples were stored at −80 °C in 10% (v/v) glycerol.

Culture-independent analysis

To assess the structure of the microbial community in the bark of *Acer palmatum*, 16S amplicon sequencing using MiSeq was performed. From the above bark suspension, five ml of the supernatant was collected by pipetting so as to minimize contamination of the bark fragments, and DNA was extracted using a Fast DNA Spin Kit (MP Biomedicals, LCC, Santa Ana, CA, USA) according to the manufacturer's instructions. The extracted DNA was adjusted with distilled water to a concentration of 30 ng/μl in a total volume of 50 μl. The DNA concentration was fluorometrically determined using Qubit Assay Kits (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a Nanophotometer (Implen GmbH, Munich, Germany). Sequence analysis of the bark samples using a MiSeq system (Illumina, Inc., San Diego, CA, USA) was performed by Fasmac Co., Ltd (Atsugi, Japan). During the first round of polymerase chain reaction (PCR) amplification, template DNA was amplified using a primer set targeting the V4 region of 16S rDNA. The hot-start PCR reaction consisted of five ng of the starting template, 10 μM of the forward primer 1st_PCR_515F (5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT—[GTG CCA GCM GCC GCG GTA A]-3′) and the reverse primer 1st_PCR_806R (5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T—[GGA CTA CHV GGG TWT CTA AT]-3′), 0.2 μl of ExTaq HS polymerase (Takara Bio Inc., Kusatsu, Japan), 1.6 μl of dNTPs, and two μl of 10× Ex Taq buffer in a total reaction volume of 20 μl. The first PCR primers included the adapter sequences for the second PCR and sequences homologous to the V4 region of 16S, as shown in parentheses. The thermal cycling profile included an initial denaturing cycle of 94 °C for 30 s, followed by 20 sequential cycles of 94 °C for 15 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension period of 72 °C for 5 min, ending with a hold cycle at 4 °C. The PCR products were purified using an Agencourt AMPure XP Kit (Beckman Coulter, Inc., Brea, CA, USA) using the manufacturer's instructions. The second PCR reaction included two μl of the purified template DNA, 10 μM of the forward primer 2nd_F (5′-[AAT GAT ACG GCG ACC ACC GAG ATC TAC AC]—[XXXXXXXX]—[ACA CTC TTT CCC TAC ACG ACG C]-3′) and the reverse primer 2nd_R (5′-[CAA GCA GAA GAC GGC ATA CGA GAT]—[YYYYYYYY]—[GTG ACT GGA GTT CAG ACG TGT G]-3′), 0.2 μl of Ex Taq HS polymerase (Takara, Kusatsu, Japan), 1.6 μl of dNTPs, and two μl of 10× Ex Taq buffer in a total reaction volume of 20 μl. The second PCR primers included the following sequences: 5′—[flow cell binding region]—[Illumina i5/i7 index]—[primer binding region (homologous to the 1st primer sequence)]—3′. The thermal cycling profile for the second PCR was a single cycle of 94 °C for 2 min, followed by eight cycles of 94 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s, with one final cycle of 72 °C for 5 min, and a hold cycle of 4 °C. The products from

the second PCR were purified using an Agencourt AMPure XP Kit. The DNA concentrations were determined using Qubit Assay Kits, and the PCR amplicons were mixed and subjected to 2×250 bp paired-end sequencing using MiSeq System v2. Cluster formation was performed using MiSeq Reagent Kit v2 and PhiX Control Kit v3, and sequence analysis was performed using MiSeq Control Software ver 2.4.1.3, Real Time Analysis ver 1.18.54 and bcl2fastq ver 1.8.4.

Analysis of the sequencing results included trimming of the primer region using Fastx toolkit, version 0.0.13.2 (*Gordon & Hannon, 2010*), joining of the forward and reverse reads using FLASH, version 1.2.10 (*Magoč & Salzberg, 2011*), and quality filtering with sickle tool, version 1.33 (*Joshi & Fass, 2011*). Filtering of the raw sequence reads was performed based on the following criteria: (1) the start region of both reads exactly matched the primer of the V4 region; (2) the minimum length was 40 bp, after the trimming of the primer region and the low-quality sequence; and (3) both reads could be joined, and the length after joining was 246–260 bp (amplicon sequence length was 285–299 bp). The 97% identity OTU clustering and chimera filtering were performed using UCHIME (USEARCH package v8.0.1623) (*Edgar et al., 2011*) in QIIME, version 1.9.0 (*Caporaso et al., 2010*). These data were then used to assign taxonomy against the Greengenes 13_8 database (*DeSantis et al., 2006*) with a 97% similarity threshold using the UCLUST v1.2.22q (*Edgar, 2010*) in the assign taxonomy script of QIIME. Details of commands and parameters are summarized in Table S1.

Isolation of bacteria

In order to examine whether previously uncultivated microorganisms could be acquired from the bark of *Acer palmatum*, cultivation was performed using a general low-nutrient agar plate medium. Bark-suspension supernatants (100 µl) were 10-fold serially diluted ($10-10^3$ fold) and were inoculated into Reasoner's 2A (R2A; Wako Pure Chemical Industries, Ltd., Osaka, Japan) culture medium that had been 10-fold diluted (DR2A) and PE03 medium (*Tamaki et al., 2005*), and incubated at 25 °C for 2 weeks under dark conditions. The strains isolated from PE03 medium are represented as Strain No. IAP and the strains isolated from DR2A medium are represented as Strain No. IAD as shown in Tables 1 and 2, respectively. In order to selectively isolate slow-growing microbes, small colonies that were visible but less than one mm in diameter were targeted. For each medium, 48 colonies were isolated based on colony color and shape. Isolated colonies were suspended in 20 µl of Tris-EDTA (TE) buffer (Sigma-Aldrich Co. LCC, St. Louis, MO, USA) for DNA extraction, in addition to preparing one ml of glycerol stock (five mM Mops, 10% (v/v) Glycerol, pH 7.0) of each isolate. The DNA samples were stored at −20 °C, and the glycerol stocks at −80 °C.

Identification of isolates

The bacterial cells suspended in TE buffer for DNA extraction were thawed, added to 20 µl of phenol:chloroform:isoamyl alcohol (25:24:1; Wako, Monza, Lombardy), and mixed by vortexing for 30 s to lyse the bacterial cells. The lysed cells were clarified by centrifugation at 15,000 rpm for 5 min, and one µl of supernatant was used as template for PCR.

Table 1 Most similar sequences of isolated microbes from PE03 medium.

Strain no.	Phylum or class	Most similar sequence	Accession no.	Similarity (%)
IAP-1	Alphaproteobacteria	Bradyrhizobium embrapense strain SEMIA 6208	AY904773	100
IAP-2	Actinobacteria	Mycobacterium peregrinum strain ATCC 14467	AF058712	97
IAP-3	Bacteroidetes	Hymenobacter terrae strain DG7A	KF862488	93
IAP-4	Actinobacteria	Amnibacterium soli strain PB243	KC251736	98
IAP-5	Actinobacteria	Amnibacterium soli strain PB243	KC251736	96
IAP-7	Gammaproteobacteria	Moraxella osloensis strain DSM 6998	AB643599	99
IAP-8	Bacteroidetes	Spirosoma spitsbergense strain SPM-9	EF451725	92
IAP-9	Alphaproteobacteria	Sphingomonas mucosissima strain CP173-2	AM229669	99
IAP-10	Actinobacteria	Actinomycetospora chibensis strain TT04-21	AB514517	98
IAP-11	Alphaproteobacteria	Psychroglaciecola arctica strain M6-76	KC511070	95
IAP-12	Actinobacteria	Actinomycetospora chlora strain TT07I-57	AB514519	97
IAP-14	Alphaproteobacteria	Afipia birgiae strain 34632	AF288304	99
IAP-15	Alphaproteobacteria	Sphingomonas mucosissima strain CP173-2	AM229669	99
IAP-16	Alphaproteobacteria	Sphingomonas asaccharolytica strain Y-345	Y09639	99
IAP-17	Actinobacteria	Pseudonocardia endophytica strain YIM 56035	DQ887489	96
IAP-18	Alphaproteobacteria	Novosphingobium barchaimii strain LL02	JN695619	98
IAP-19	Alphaproteobacteria	Novosphingobium barchaimii strain LL02	JN695619	98
IAP-20	Alphaproteobacteria	Sphingomonas hankookensis strain ODN7	FJ194436	98
IAP-21	Actinobacteria	Microbacterium fluvii strain YSL3-15	AB286028	97
IAP-23	Actinobacteria	Cellulomonas pakistanensis strain NCCP-11	AB618146	97
IAP-24	Actinobacteria	Cellulomonas pakistanensis strain NCCP-11	AB618146	97
IAP-27	Betaproteobacteria	Variovorax paradoxus strain NBRC 15149	AB680784	99
IAP-28	Betaproteobacteria	Variovorax guangxiensis strain GXGD002	JF495126	99
IAP-29	Actinobacteria	Jatrophihabitans huperziae strain I13A-01604	KR184574	91
IAP-30	Actinobacteria	Lysinimonas soli strain SGM3-12	JN378395	98
IAP-31	Actinobacteria	Amnibacterium kyonggiense strain KSL51201-037	FJ527819	96
IAP-32	Alphaproteobacteria	Phenylobacterium aquaticum strain W2-3-4	KT309087	94
IAP-33	Acidobacteria	Terriglobus roseus strain KBS 63	DQ660892	99
IAP-35	Actinobacteria	Microbacterium saccharophilum strain K-1	AB736273	96
IAP-36	Alphaproteobacteria	Sphingomonas koreensis strain NBRC 16723	AB681117	98
IAP-37	Bacteroidetes	Flavobacterium rivuli strain WB3.3-2	AM934661	93
IAP-39	Actinobacteria	Microbacterium saccharophilum strain K-1	AB736273	97
IAP-40	Alphaproteobacteria	Brevundimonas albigilva strain NHI-13	KC733808	95
IAP-41	Actinobacteria	Microlunatus panaciterrae strain Gsoil 954	AB271051	96
IAP-42	Actinobacteria	Microbacterium saccharophilum strain K-1	AB736273	96
IAP-45	Bacteroidetes	Mucilaginibacter rigui strain NBRC 101115	AB681382	96
IAP-46	Actinobacteria	Nakamurella multipartita strain DSM 44233	CP001737	94
IAP-47	Actinobacteria	Microbacterium fluvii strain YSL3-15	AB286028	97
IAP-48	Alphaproteobacteria	Sphingomonas asaccharolytica strain Y-345	Y09639	98

Template DNA was amplified with an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using 0.25 μl of TaKaRa Ex Taq (five U/μl), five μl of 10× Ex Taq Buffer, four μl of dNTP mix (Takara, Kusatsu, Japan), and 50 pmol of primers 8F (*Weisburg et al., 1991*;

Table 2 Most similar sequences of isolated microbes from DR2A medium.

Strain no.	Phylum or class	Most similar sequence	Accession no.	Similarity (%)
IAD-1	*Bacteroidetes*	*Mucilaginibacter rigui* strain NBRC 101115	AB681382	96
IAD-2	*Actinobacteria*	*Actinomycetospora cinnamomea* strain IY07-53	AB514520	97
IAD-3	*Bacteroidetes*	*Spirosoma panaciterrae* strain Gsoil 1519	EU370956	90
IAD-4	*Actinobacteria*	*Microbacterium fluvii* strain YSL3-15	AB286028	97
IAD-5	*Bacteroidetes*	*Spirosoma spitsbergense* strain SPM-9	EF451725	90
IAD-6	*Actinobacteria*	*Nocardioides islandensis* strain MSL 26	EF466123	99
IAD-7	*Betaproteobacteria*	*Ramlibacter ginsenosidimutans* strain BXN5-27	EU423304	96
IAD-9	*Actinobacteria*	*Nocardioides halotolerans* strain MSL-23	EF466122	98
IAD-10	*Bacteroidetes*	*Spirosoma fluminis* strain 15J17	LC148305	91
IAD-11	*Alphaproteobacteria*	*Sphingopyxis wooponensis* strain 03SU3-P	HQ436493	94
IAD-12	*Actinobacteria*	*Microbacterium saccharophilum* strain K-1	AB736273	97
IAD-13	*Actinobacteria*	*Nocardioides halotolerans* strain MSL-23	EF466122	98
IAD-14	*Alphaproteobacteria*	*Methylobacterium dankookense* strain SW08-7	FJ155589	97
IAD-15	*Actinobacteria*	*Nocardioides soli* strain mbc-2	JF937914	93
IAD-19	*Alphaproteobacteria*	*Sphingopyxis wooponensis* strain 03SU3-P	HQ436493	95
IAD-21	*Abditibacteriota*	*Abditibacterium utsteinense* strain R-68213	KY386500	91
IAD-24	*Firmicutes*	*Staphylococcus hominis* subsp. *Novobiosepticus* strain GTC 1228	AB233326	99
IAD-28	*Bacteroidetes*	*Mucilaginibacter soli* strain R9-65	JF701183	96
IAD-29	*Alphaproteobacteria*	*Sphingomonas mucosissima* strain CP173-2	AM229669	99
IAD-30	*Actinobacteria*	*Microbacterium saccharophilum* strain K-1	AB736273	96
IAD-31	*Bacteroidetes*	*Fibrella aestuarina* strain BUZ 2	HE796683	86
IAD-32	*Alphaproteobacteria*	*Amaricoccus kaplicensis* strain Ben101	U88041	94
IAD-33	*Alphaproteobacteria*	*Sphingopyxis wooponensis* strain 03SU3-P	HQ436493	95
IAD-34	*Alphaproteobacteria*	*Sphingomonas asaccharolytica* strain Y-345	Y09639	98
IAD-37	*Alphaproteobacteria*	*Sphingomonas hankookensis* strain ODN7	FJ194436	99
IAD-41	*Actinobacteria*	*Cellulomonas pakistanensis* strain NCCP-11	AB618146	97
IAD-42	*Bacteroidetes*	*Spirosoma fluminis* strain 15J17	LC148305	91
IAD-43	*Actinobacteria*	*Cellulomonas pakistanensis* strain NCCP-11	AB618146	96
IAD-44	*Betaproteobacteria*	*Ramlibacter ginsenosidimutans* strain BXN5-27	EU423304	96
IAD-45	*Alphaproteobacteria*	*Methylobacterium brachythecii* strain 99b	AB703239	99
IAD-48	*Actinobacteria*	*Nocardioides halotolerans* strain MSL-23	EF466122	98

5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (*Lane, 1991*; 5′-TAC GGY TAC CTT GTT ACG ACT T-3′) in a 50-μl reaction. The thermal cycling profile was one cycle at 94 °C for 20 s, 30 sequential cycles of 94 °C for 20 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min and a hold at 4 °C. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The concentration of the purified DNA was determined using a V-730BIO Spectrophotometer (JASCO Corporation, Tokyo, Japan). A 100-ng aliquot of PCR-amplified DNA and 7.5 pmol of 8F primer were mixed in a total volume of 15 μl and analyzed by Sanger sequencing by Takara Bio Inc. (Kusatsu, Japan). In general, the sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit

(Takara, Kusatsu, Japan) and an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequence reads obtained were compared with those in the NCBI database of rRNA type strains/prokaryotic 16S ribosomal RNA (database of bacterial and archaeal type strains, except environmental clones, hereafter referred to as type strains) or nucleotide collection using the BLAST program. Taxonomic classification at the genus level was performed using RDP Classifier as previously described (*Wang et al., 2007*).

Phylogenetic analysis of strain IAD-21

BLAST searches of partial 16S rDNA sequences indicated that the sequence similarity of the strain IAD-21, isolated from DR2A medium, with type strains in the database was relatively low at 91%. Part of the molecular phylogenetic analysis based on 16S rDNA sequence for strain IAD-21 was performed by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). The DNA was extracted from the IAD-21 bacterial cells using a crude preparation of the lytic enzyme Achromopeptidase® (Wako, Monza, Lombardy) and PCR amplified using PrimeSTAR HS DNA Polymerase (Takara, Kusatsu, Japan) with primers 9F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1541R (5′-AAG GAG GTG ATC CAG CC-3′) (*Nakagawa & Kawasaki, 2001*). Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) and an ABI PRISM 3130 xl Genetic Analyzer System (Applied Biosystems, Waltham, MA, USA) with primers 9F, 785F (5′-GGA TTA GAT ACC CTG GTA GTC-3′), 802R (5′-TAC CAG GGT ATC TAA TCC-3′), and 1541R. The precise nucleotide sequence was determined with ChromasPro 1.7 (Technelysium, South Brisbane, QLD, Australia). The full-length reads of 16S rDNA sequence obtained (about 1,500 bp) were compared with sequences in DB-BA 12.0 (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan) and international nucleotide sequence databases, including the DNA Data Bank of Japan, the European Nucleotide Archive, and GenBank (DDBJ/ENA(EMBL)/GenBank) using the TechnoSuruga Lab Microbial identification system (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan). Since the full length of the 16S rDNA sequence of strain IAD-21 showed high similarity with clones derived from *Abditibacteriota*/candidate division FBP (*Lee et al., 2013*), some 16S rDNA sequences of *Abditibacteriota* and some bacterial phyla (*Deinococcus-Thermus*, *Armatimonadetes*, *Chloroflexi*, and candidate division WS1) were obtained from the database and subjected to molecular phylogenetic analysis (Table S2). Following multiple-sequence alignment by CLUSTAL W (*Thompson, Higgins & Gibson, 1994*), the alignment was edited with BioEdit, version 7.2.5 (*Hall, 1999*). Phylogenetic trees were constructed by the neighbor-joining (NJ) (*Saitou & Nei, 1987*) method and the maximum likelihood (ML) (*Felsenstein, 1981*) method using the MEGA X software (*Kumar et al., 2018*). The Kimura 2-parameter model (*Kimura, 1980*) for the NJ tree and the Tamura-Nei model (*Tamura & Nei, 1993*) with the gamma distributed with invariant sites (G+I) model (*Gu, Fu & Li, 1995*) for the ML tree were employed. Bootstrap values (*Felsenstein, 1985*) were determined from 1,000 re-samplings.

Nucleotide accession number

Culture-independent MiSeq sequence reads of the 16S rDNA have been deposited in the DDBJ sequence read archive (DRA) under accession numbers DRA006430 and DRA008228. Sequence reads of 16S rDNA from the bacterial isolates have been deposited in the DDBJ nucleotide sequences databank under accession numbers LC361357–LC361426.

RESULTS

By analyzing the microbial community structure of *Acer palmatum* bark using MiSeq, a total of 97,288 reads were detected. In total, 4,560 OTUs were defined with 97% sequence similarity. The phylogenetic distribution of the defined OTUs at the phylum level is shown in Fig. 1A. Sequence reads belonging to 27 bacterial phyla were detected from the *Acer palmatum* bark. *Proteobacteria*, at 34.9%, was the most abundant bacterial phylum, followed by 26.2% for *Bacteroidetes*, and 9.2% for *Acidobacteria*. In addition, *Gemmatimonadetes* (5.3%), *Verrucomicrobia* (4.0%), and *Armatimonadetes* (1.1%) were also detected, of which many bacteria were uncultured. *Abditibacteriota* (0.7%) was also detected at a relatively low abundance. In order to confirm whether the above phyla could be universally detected from *Acer palmatum* bark, three samples were collected from two individual trees (one of them being the previously analyzed tree), and re-analysis of the 16S amplicon sequencing was performed (Fig. 2; Table S3). A total of 51,109–77,943 reads were detected, and 673–1,794 OTUs were defined with 97% sequence similarity. These phyla (*Acidobacteria*: 6.7–33.1%, *Gemmatimonadetes*: 0.1–1.3%, *Verrucomicrobia*: 1.9–9.1%, *Armatimonadetes*: 0.4–1.4%) and *Abditibacteriota* (0.04–0.7%), although varying in abundance, were also detected in the re-analysis (Fig. 2). Consistently, *Proteobacteria* was the most abundant phylum in all samples, although the ranks in the lower abundances were quite variable. Thus, both candidate divisions and rarely cultivated groups were found in the *Acer palmatum* bark.

The top 30 genera, found in the *Acer palmatum* bark based on MiSeq analysis, are shown in Table S3. *Sphingomonas*, *Actinomycetospora*, unidentified genus in *Chitinophagaceae*, unidentified genus in *Sphingomonadaceae*, unidentified genus in *Methylocystaceae*, and unidentified genus in *Acetobacteraceae* were commonly detected in the top 30 genera in all seven samples. It was suggested that these genera universally inhabit *Acer palmatum* bark. Furthermore, some genera belonging to phyla *Acidobacteria*, *Verrucomicrobia*, *Gemmatimonadetes*, and *Armatimonadetes* were among the top 30 at the genus-level in terms of relative abundance. These results revealed that many uncultured bacteria inhabited the *Acer palmatum* bark.

To determine whether these microorganisms could be cultured and isolated, we incubated *Acer palmatum* bark for 2 weeks in DR2A or PE03 agar medium. A large number of colonies were obtained on both media. Among the 96 isolated strains (48 strains isolated from each medium), there were nine strains from the PE03 medium and 17 strains from the DR2A medium that could not be sub-cultured. The remaining 39 strains from the PE03 medium and 31 strains from the DR2A medium could be sub-cultured and were subjected to sequence analysis. The phylogenetic distribution of the isolated strains at the
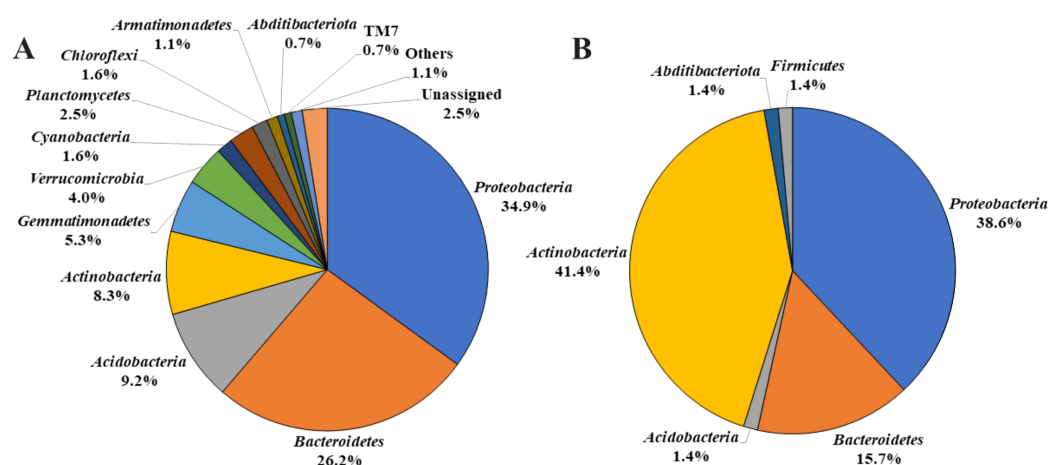
Figure 1 Bacterial phyla detected from *A. palmatum* bark. Relative abundances of bacterial phyla detected from *Acer palmatum* bark. (A) Results from the analysis of 16S rDNA sequences detected by culture-independent evaluation of bark using next generation sequencing with a MiSeq system. (B) Isolates obtained by culture-dependent analysis using PE03 and DR2A agar media, and sequenced by Sanger method followed by sequence alignment and characterization analyses.

phylum level is shown in Fig. 1B. Of the isolated strains, *Actinobacteria* was the most frequent with 29 strains (41.4%), followed by *Proteobacteria* with 27 strains (38.6%), and *Bacteroidetes* with 11 strains (15.7%). Compared with the results of MiSeq analysis, the results from the isolation analysis differed in the relative abundance at the phylum level. Compared with the top 30 genera detected by MiSeq analysis from the seven samples (Table S3), the genera that could be cultured and isolated had three genera in common (*Sphingomonas*, an unidentified genus in *Sphingomonadaceae*, and *Actinomycetospora*). Genus-level relative abundance was low for most of the cultured and isolated genera detected in the bark. For example, the relative abundances of *Novosphingobium* (0–0.03%) and *Nocardioides* (0.001–0.21%) in the *Acer palmatum* bark were very low. The results of culture-independent analysis do not always accurately reflect the actual microbial community structure in the bark due to variation among taxa in DNA extraction efficiency, 16S copy number variation and bias of universal primers. However, this suggested that whether microorganisms in the bark could be cultured or not was not predictable based on the relative abundance in the bark.

The classification at the genus level of all isolated strains obtained on the two types of medium is shown in Table 3. *Sphingomonas* was the most frequently isolated genus in this study with nine strains, followed by *Microbacterium* with five strains, *Spirosoma* with four strains, an unclassified genus in *Cellulomonadaceae* with four strains, and an unclassified genus in *Nocardioidaceae* with four strains. In addition, a bacterial strain belonging to *Acidobacteria*, which is difficult to culture and has very few isolated strains (*Eichorst, Kuske & Schmidt, 2011*; *Navarrete et al., 2013*; *Tanaka et al., 2017*), was isolated on PE03 medium, and based on classifier and BLAST analysis was presumed to belong to the genus *Terriglobus*.
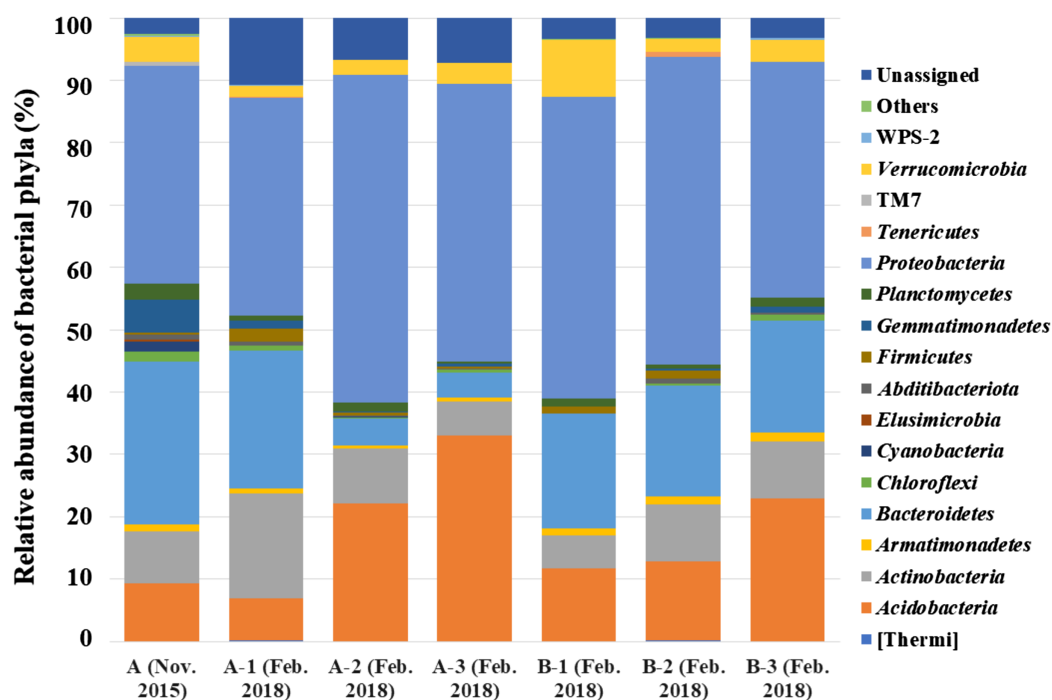
Figure 2 **Bacterial phyla detected from seven samples collected from two *A. palmatum* trees.** Relative abundance of bacterial phyla detected from seven samples collected from two *A. palmatum* trees. Results from the analysis of 16S rDNA sequences detected by culture-independent evaluation of bark using next generation sequencing with a MiSeq system. A and B refer to the tree number, and 1–3 refer to biological replicate within a single tree. Sample A (Nov. 2015) is identical to that in Fig. 1A.

According to *Tamaki et al. (2009)*, the isolates were phylogenetically divided into two groups on the basis of their partial 16S rDNA sequence similarities to the reference sequences in the public databases: (i) ≤97% similarity to type strains: isolates with high phylogenetic novelty, and (ii) >97% similarity: isolates with low phylogenetic novelty. These criteria were used as objective indicators of the phylogenetic novelty of isolates, although they do not necessarily indicate taxonomic novelty at the genus or species level (*Tamaki et al., 2009*). The culture collection obtained from this study included 44 strains (62.8% of the total) that showed ≤97% 16S rDNA sequence similarity with type strains (Tables 1 and 2). However, since only partial 16S rDNA sequences (V1-V3 regions) were used, the resolution might be limited compared to full length 16S rDNA similarity searches. We focused on one particular isolate, strain IAD-21, a potentially novel microorganism, cultured on DR2A medium (Table 2). The full-length 16S rDNA sequence for IAD-21 was determined, and its sequence similarity with type strains was confirmed. We conducted a BLAST search including environmental clones and found high sequence similarity with *Abditibacterium utsteinense* R-68213T (Table 2) and clones belonging to *Abditibacteriota*/candidate division FBP, including clone UMAB-cl-090 obtained from the Antarctic soil (sequence similarity 95.2%; accession number FR749715), clone ncd242h05c1 obtained from human volar forearm skin (sequence similarity 97.2%; accession number HM269099), and clone ncd1960f07c1 obtained from human

Table 3 Taxonomic classification of isolates obtained by culture-dependent analysis on the basis of classifier program.

Phylum	Class	Order	Family	Genus	Number of isolates	
					PE03	DR2A
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Unclassified	2	
		Rhizobiales	Bradyrhizobiaceae	Unclassified	2	
			Methylobacteriaceae	Methylobacterium		2
			Unclassified	Unclassified	1	
		Rhodobacterales	Rhodobacteraceae	Unclassified		1
		Sphingomonadales	Sphingomonadaceae	Novosphingobium	2	
				Sphingomonas	6	3
				Unclassified		3
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ramlibacter		1
				Variovorax	2	
				Unclassified		1
	Gammaproteobacteria	Rhodospirillales	Unclassified Rhodospirillales	Enhydrobacter	1	
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Fibrella		1
				Spirosoma	1	3
				Unclassified		1
			Hymenobacteraceae	Hymenobacter	1	
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	1	
	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	1	2
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae	Terriglobus	1	
Actinobacteria	Actinobacteria	Actinomycetales	Unclassified	Unclassified	2	
		Corynebacteriales	Mycobacteriaceae	Mycobacterium	1	
		Micrococcales	Cellulomonadaceae	Unclassified	2	2
			Microbacteriaceae	Amnibacterium	3	
				Lysinimonas	1	
				Microbacterium	3	2
				Unclassified	2	1
		Propionibacteriales	Nocardioidaceae	Nocardioides		1
				Unclassified		4
			Propionibacteriaceae	Microlunatus	1	
		Pseudonocardiales	Pseudonocardiaceae	Actinomycetospora	2	1
				Pseudonocardia	1	
Abditibacteriota	Unidentified	Unidentified	Unidentified	Unidentified		1
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		1
Total isolate number					39	31

antecubital fossa skin (sequence similarity 96.9%; accession number JF171142) (*Lee et al., 2013*). Since the full length of the 16S rDNA sequence from strain IAD-21 showed high sequence similarity with sequences belonging to *Abditibacteriota*, we obtained 16S rDNA sequences from *Armatimonadetes*, which is considered to be phylogenetically close to *Abditibacteriota*, and from *Chloroflexi* and *Deinococcus-Thermus* according to *Tahon et al. (2018)*. Sequences of the top 100 hits from the BLAST search of strain IAD-21,

and sequences used for generating the phylogenetic tree of *Abditibacteriota*/candidate division FBP and WS1 from *Lee et al. (2013)*, were subjected to molecular phylogenetic analysis (Table S2). As a result, it was suggested that strain IAD-21 belonged to the same cluster as sequences from the phylum *Abditibacteriota* (Fig. 3; Fig. S1).

DISCUSSION

Although many studies are being carried out on the microbial community structure associated with the tree phyllosphere or rhizosphere, there are few reports that focus on the microbial community structure existing in the bark. In this study, we performed culture-independent analysis using MiSeq and isolation experiments to investigate the microbial community structure existing in *Acer palmatum* bark.

As a result of cultivation and isolation experiments, *Microbacterium*, an unclassified genus in *Cellulomonadaceae*, an unclassified genus in *Microbacteriaceae*, an unclassified genus in *Nocardioidaceae* (all belonging to *Actinobacteria*), and *Sphingomonas* were frequently isolated in this study. Microorganisms from numerous closely related genera have also been detected in culture-dependent and -independent analyses of other barks and branches including elm, poplar, grapevine, *Acer negundo*, *Acer pseudoplatanus*, and *G. biloba* (*Mocali et al., 2003*; *Ulrich, Ulrich & Ewald, 2007*; *Martins et al., 2013*; *Shen & Fulthorpe, 2015*; *Leff et al., 2015*; *Aschenbrenner et al., 2017*), and are considered to be the natural inhabitants of bark. To the best of our knowledge, there are no prior reports of *Spirosoma* being cultured and isolated from bark. Since the chemical constituents of the bark differ depending on the tree species (*Feng et al., 2013*), it is considered that the bark of different tree species would have different microbial communities. In the future, more detailed analysis of microbial community structure in the bark will be required with respect to changes in the community depending on the tree species and localization of microorganisms in bark organs, using both culture-dependent and -independent analyses. Through the current culture-dependent analysis, a wide range of microbial species was identified.

By culture-independent analysis of *Acer palmatum* bark, members of rarely cultivated phyla such as *Acidobacteria*, *Armatimonadetes*, *Verrucomicrobia*, and *Gemmatimonadetes* were detected. This is consistent with other culture-independent analyses of the microbial community structures of bark samples. For instance, *Acidobacteria* and *Verrucomicrobia* were detected in samples from *Acer pseudoplatanus* bark at frequencies of 10.7% and 4.0%, respectively (*Aschenbrenner et al., 2017*), and *Acidobacteria* and *Armatimonadetes* were detected from *G. biloba* bark samples at 13.1% and 1.0%, respectively (*Leff et al., 2015*). Since these exist universally in soil environments (*Bergmann et al., 2011*; *DeBruyn et al., 2011*; *Lee, Dunfield & Stott, 2014*; *Kielak et al., 2016*), it is believed that they are spread by means such as the wind or insects and colonize the bark. Although *Acidobacteria* are believed to be as environmentally widespread as *Proteobacteria* (*Barns, Takala & Kuske, 1999*), many of them are slow growing and oligotrophic bacteria that are largely comprised of uncultured taxa (*Da Rocha, Van Overbeek & Van Elsas, 2009*; *Ward et al., 2009*). *Leff et al. (2015)* suggested that old bark
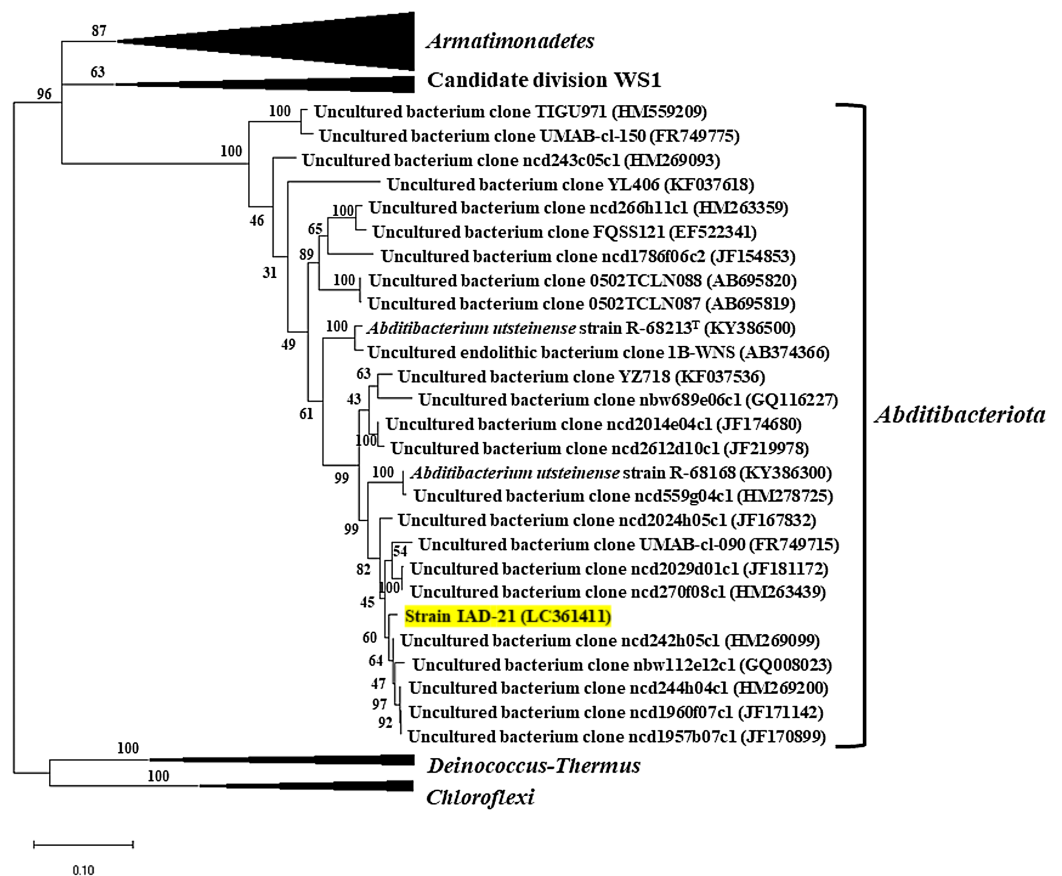
Figure 3 Phylogenetic tree of strain IAD-21. Phylogenetic tree of strain IAD-21 and related sequences of *Abditibacteriota* based on 16S rDNA. In part, the full-length reads of 16S rDNA sequence were compared to sequences in international nucleotide sequence databases including the DNA Data Bank of Japan, the European Nucleotide Archive, and GenBank (DDBJ/ENA/GenBank). The phylogenetic tree was constructed using the maximum likelihood method and the Tamura-Nei model with the gamma distributed with invariant sites (G+I) model for estimating nucleotide substitutions. Bootstrap values were determined from 1,000 re-samplings. The newly identified and unique strain IAD-21 is located within the *Abditibacteriota* cluster. The scale is given below the phylogenetic tree.

environments provide more suitable locales for stable inhabitation over long periods of time for slow-growing and oligotrophic bacteria such as *Acidobacteria* than do the leaf or branch environments. In addition, there is little disturbance from UV radiation or precipitation in the old bark environment. They cited this limited disturbance in the old bark as a factor for the richness of the microbial community in *G. biloba* bark and for the detection of rarely cultivated phyla. In a few reports from analyses of the microbial community structure of bark, it was stated that comparison of results from previous work was difficult due to the scarcity of available data (*Leff et al., 2015*). In reviewing the results from this study, we agree with this impression. While comparisons may be difficult, it is still possible to speculate. In addition, in the outer bark consisting of dead cells, light irradiation and symbiosis with lichens and cyanobacteria may occur, and in the inner bark consisting of living cells, flow of photosynthates may affect the symbiotic microbial
community (*Baldrian, 2017*). It is possible that the long-term existence of these factors in a stable environment may promote the growth of a microorganism on the bark.

Martins et al. (2013) also reported the high diversity of acquired microorganisms in the bark as compared with that in organs such as leaves and fruits of grapevine. Recently in the bark tissue, microorganisms belonging to *Acidobacteria* (*Yamada et al., 2014*) and *Armatimonadetes* (*Li, Kudo & Tonouchi, 2018*) that are difficult-to-cultivate and slow-growing taxa were isolated. It can be inferred that the bark environment is a convenient residence for such bacterial taxa. Moreover, the fact that the bark harbors diverse microbial communities may have some meaning for trees. According to *Khorsandy et al. (2016)*, the frequency of fungal endophytes in the bark of *Platanus orientalis* L. was significantly greater in older trees (60.04%) than in younger ones (39.96%). Existence of such fungal endophytes was positively correlated with the iron and potassium concentrations of the leaves, tree height, circumference, and improved visual appearance. These results suggested that fungal endophytes enhanced nutrient assimilation in trees, at least partly contributing to increased survival of the older trees (*Khorsandy et al., 2016*). Thus, there is no denying that old bark may benefit by harboring diverse microbial communities. However, since *Khorsandy et al. (2016)* reported fungal endophytes, while *Leff et al. (2015)* and *Martins et al. (2013)* referred to bacterial epiphytes, it is necessary to gain an understanding of the microbial community structure (both fungal and bacterial) of each organ of the bark area. It has been reported that members of *Acidobacteria* contribute to increases in biomass, rhizosphere morphology changes, production of indole-3-acetic acid, and iron absorption in *Arabidopsis thaliana* (*Kielak, Cipriano & Kuramae, 2016*). Further isolation and cultivation of microbes from phyla like *Acidobacteria*, which contain a considerable number of uncultured microbes, will lead to a better understanding of the tree-microbiota symbiotic system.

In this study, we successfully isolated a novel microorganism, strain IAD-21. Based on molecular phylogenetic analyses, it was suggested that IAD-21 belongs to *Abditibacteriota*. In addition to bark, *Abditibacteriota* has been detected by culture-independent analysis of Antarctic soil (*Tytgat et al., 2016*), with two strains belonging to this division isolated from Antarctic soil (*Tahon & Willems, 2017*). One of them, strain R-68213, has been officially described as the first cultivated representative of the phylum *Abditibacteriota* and renamed as *Abditibacterium utsteinense* strain R-68213T (*Tahon et al., 2018*). These strains were successfully isolated by mimicking the Antarctic environment, using a low-nutrient medium for phototrophic bacteria, and adjusting the photoperiod over 10 weeks. However, in the current study, strain IAD-21 was relatively easy to culture, as we succeeded in its isolation by simply using a general low-nutrient medium during a 2-week cultivation period. In addition, isolated strains from *Acer palmatum* bark were relatively novel, even if they belonged to taxa with high cultivation frequencies. The results of subsequent experiments exhibited the high phylogenetic novelty of isolates from *Acer palmatum* bark (Table S4). Furthermore, it is necessary to identify the reasons for obtaining high phylogenetic novelty of isolated strains and why strain IAD-21 could be cultivated with ease. One probable cause for the easy cultivation of strain IAD-21 could be its ability to grow in the relatively stable, less disturbed and unexplored

environment of the bark. It is desirable that the culture efficiency be evaluated by the performance of comprehensive cultivation and isolation of microorganisms from the bark, and that the relationship between the poorly cultivated microorganisms and the tree bark be clarified.

CONCLUSIONS

Based on our study, we propose that *Acer palmatum* bark might prove to be a promising source of novel microorganisms. Since the culture conditions used in this study were relatively non-exceptional and only involved using low-nutrient media, it is possible that additional microorganisms could be acquired utilizing special culture methods and conditions (e.g., modification of the culture substrate, gelling agents, and medium composition). Analysis of the microbial community structure of various tree species and the isolation of uncultured microorganisms may lead to a more comprehensive understanding of the yet uncharacterized tree-microbiota symbiotic system. Microorganisms from bark samples may also be important from an academic point of view to understand microbial ecology, and further research is expected to clarify the unknown sectors of the microbial phylogenetic tree.

ACKNOWLEDGEMENTS

The manuscript has been edited carefully by native-English-speaking professional editor from Editage, a division of Cactus Communications. Collection of plant samples was carried out with permission from the Ichimura Foundation for New Technology Botanical Research Gardens as a research collaborator.

Funding

Kazuki Kobayashi was supported by the Futaba Electronics Memorial Foundation, Japan in the form of a doctoral program scholarship. This work was also supported by Japan Society for the Promotion of Science KAKENHI Challenging Research (Exploratory, grant number 17K19218), the Ichimura Foundation for New Technology, Japan, and the Sumitomo Electric Industries Group Corporate Social Responsibility Foundation, Japan (grants to Hideki Aoyagi). There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Futaba Electronics Memorial Foundation, Japan.
Japan Society for the Promotion of Science KAKENHI Challenging Research (Exploratory): 17K19218.
Ichimura Foundation for New Technology, Japan, and the Sumitomo Electric Industries Group Corporate Social Responsibility Foundation, Japan (grants to Hideki Aoyagi).

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Kazuki Kobayashi conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Hideki Aoyagi conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft, supervised the research.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

Culture-independent MiSeq sequence reads of the 16S rDNA are available in the DDBJ sequence read archive (DRA): DRA006430 and DRA008228.

Sequence reads of 16S rDNA from the bacterial isolates are available in the DDBJ nucleotide sequences databank: LC361357–LC361426 and LC490821–LC490862.

Data Availability

The raw data are available as Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7876#supplemental-information.

REFERENCES

Aschenbrenner IA, Cernava T, Erlacher A, Berg G, Grube M. 2017. Differential sharing and distinct co-occurrence networks among spatially close bacterial microbiota of bark, mosses and lichens. *Molecular Ecology* **26(10)**:2826–2838 DOI 10.1111/mec.14070.

Baldrian P. 2017. Forest microbiome: diversity, complexity and dynamics. *FEMS Microbiology Reviews* **41(2)**:109–130 DOI 10.1093/femsre/fuw040.

Barns SM, Takala SL, Kuske CR. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Applied and Environmental Microbiology* **65(4)**:1731–1737.

Berendsen RL, Pieterse CMJ, Bakker PAHM. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* **17(8)**:478–486 DOI 10.1016/j.tplants.2012.04.001.

Bergmann GT, Bates ST, Eilers KG, Lauber CL, Caporaso JG, Walters WA, Knight R, Fierer N. 2011. The under-recognized dominance of *Verrucomicrobia* in soil bacterial communities. *Soil Biology and Biochemistry* **43(7)**:1450–1455 DOI 10.1016/j.soilbio.2011.03.012.

Bodenhausen N, Bortfeld-Miller M, Ackermann M, Vorholt JA. 2014. A synthetic community approach reveals plant genotypes affecting the phyllosphere microbiota. *PLOS Genetics* **10(4)**:e1004283 DOI 10.1371/journal.pgen.1004283.

Buck JW, Lachance M-A, Traquair JA. 1998. Mycoflora of peach bark: population dynamics and composition. *Canadian Journal of Botany* **76(2)**:345–354 DOI 10.1139/b98-009.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7(5)**:335–336 DOI 10.1038/nmeth.f.303.

Comby M, Lacoste S, Baillieul F, Profizi C, Dupont J. 2016. Spatial and temporal variation of cultivable communities of co-occurring endophytes and pathogens in wheat. *Frontiers in Microbiology* **7**:403 DOI 10.3389/fmicb.2016.00403.

Da Rocha UN, Van Overbeek L, Van Elsas JD. 2009. Exploration of hitherto-uncultured bacteria from the rhizosphere. *FEMS Microbiology Ecology* **69(3)**:313–328 DOI 10.1111/j.1574-6941.2009.00702.x.

DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M. 2011. Global biogeography and quantitative seasonal dynamics of *Gemmatimonadetes* in soil. *Applied and Environmental Microbiology* **77(17)**:6295–6300 DOI 10.1128/AEM.05005-11.

Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, Von Mering C, Vorholt JA. 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **106(38)**:16428–16433 DOI 10.1073/pnas.0905240106.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* **72(7)**:5069–5072 DOI 10.1128/AEM.03006-05.

Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26(19)**:2460–2461 DOI 10.1093/bioinformatics/btq461.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27(16)**:2194–2200 DOI 10.1093/bioinformatics/btr381.

Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences of the United States of America* **112(8)**:E911–E920 DOI 10.1073/pnas.1414592112.

Eichorst SA, Kuske CR, Schmidt TM. 2011. Influence of plant polymers on the distribution and cultivation of bacteria in the phylum *Acidobacteria*. *Applied and Environmental Microbiology* **77(2)**:586–596 DOI 10.1128/AEM.01080-10.

Felsenstein J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17(6)**:368–376 DOI 10.1007/BF01734359.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39(4)**:783–791 DOI 10.1111/j.1558-5646.1985.tb00420.x.

Feng S, Cheng S, Yuan Z, Leitch M, Xu CC. 2013. Valorization of bark for chemicals and materials: a review. *Renewable and Sustainable Energy Reviews* **26**:560–578 DOI 10.1016/j.rser.2013.06.024.

Filteau M, Lagacé L, LaPointe G, Roy D. 2010. Seasonal and regional diversity of maple sap microbiota revealed using community PCR fingerprinting and 16S rRNA gene clone libraries. *Systematic and Applied Microbiology* **33(3)**:165–173 DOI 10.1016/j.syapm.2010.02.003.

Gordon A, Hannon GJ. 2010. Fastx-toolkit. FASTQ/A short-reads preprocessing tools. *Available at* http://hannonlab.cshl.edu/fastx_toolkit (accessed 13 January 2018).

Gu X, Fu YX, Li WH. 1995. Maximum likelihood estimation of the heterogeneity of substitution rate among nucleotide sites. *Molecular Biology and Evolution* **12(4)**:546–557 DOI 10.1093/oxfordjournals.molbev.a040235.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**:95–98.

Hammond DH, Varner JM, Kush JS, Fan Z. 2015. Contrasting sapling bark allocation of five southeastern USA hardwood tree species in a fire prone ecosystem. *Ecosphere* **6(7)**:112 DOI 10.1890/ES15-00065.1.

Joshi NA, Fass JN. 2011. Sickle: a sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33) [Software]. *Available at https://github.com/najoshi/sickle* (accessed 13 January 2018).

Kallmeyer J, Pockalny R, Adhikari RR, Smith DC, D'Hondt S. 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. *Proceedings of the National Academy of Sciences of the United States of America* **109(40)**:16213–16216 DOI 10.1073/pnas.1203849109.

Khorsandy S, Nikbakht A, Sabzalian MR, Pessarakli M. 2016. Effect of fungal endophytes on morphological characteristics, nutrients content and longevity of plane trees (*Platanus orientalis* L.). *Journal of Plant Nutrition* **39(8)**:1156–1166 DOI 10.1080/01904167.2015.1109113.

Kielak AM, Barreto CC, Kowalchuk GA, Van Veen JA, Kuramae EE. 2016. The ecology of *Acidobacteria*: moving beyond genes and genomes. *Frontiers in Microbiology* **7(156341)**:744 DOI 10.3389/fmicb.2016.00744.

Kielak AM, Cipriano MAP, Kuramae EE. 2016. *Acidobacteria* strains from subdivision 1 act as plant growth-promoting bacteria. *Archives of Microbiology* **198(10)**:987–993 DOI 10.1007/s00203-016-1260-2.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16(2)**:111–120 DOI 10.1007/BF01731581.

Köberl M, Schmidt R, Ramadan EM, Bauer R, Berg G. 2013. The microbiome of medicinal plants: diversity and importance for plant growth, quality, and health. *Frontiers in Microbiology* **4**:400 DOI 10.3389/fmicb.2013.00400.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35(6)**:1547–1549 DOI 10.1093/molbev/msy096.

Laforest-Lapointe I, Messier C, Kembel SW. 2016a. Host species identity, site and time drive temperate tree phyllosphere bacterial community structure. *Microbiome* **4(1)**:27 DOI 10.1186/s40168-016-0174-1.

Laforest-Lapointe I, Messier C, Kembel SW. 2016b. Tree phyllosphere bacterial communities: exploring the magnitude of intra- and inter-individual variation among host species. *PeerJ* **4(12)**:e2367 DOI 10.7717/peerj.2367.

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic Acid Techniques in Bacterial Systematics*. Chichester: John Wiley and Sons, 115–175.

Ledford H. 2015. Promising antibiotic discovered in microbial 'dark matter'. *Nature News* **76**:2445 DOI 10.1038/nature.2015.16675.

Lee KCY, Dunfield PF, Stott MB. 2014. The phylum *Armatimonadetes*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, eds. *The Prokaryotes*. Berlin, Heidelberg: Springer, 447–458.

Lee KCY, Herbold CW, Dunfield PF, Morgan XC, McDonald IR, Stott MB. 2013. Phylogenetic delineation of the novel phylum *Armatimonadetes* (former candidate division OP10) and

definition of two novel candidate divisions. *Applied and Environmental Microbiology* **79(7)**:2484–2487 DOI 10.1128/AEM.03333-12.

Leff JW, Tredici PD, Friedman WE, Fierer N. 2015. Spatial structuring of bacterial communities within individual *Ginkgo biloba* trees. *Environmental Microbiology* **17(7)**:2352–2361 DOI 10.1111/1462-2920.12695.

Li J, Kudo C, Tonouchi A. 2018. *Capsulimonas corticalis* gen. nov., sp. nov., an aerobic capsulated bacterium, of a novel bacterial order, *Capsulimonadales* ord. nov., of the class *Armatimonadia* of the phylum *Armatimonadetes*. *International Journal of Systematic and Evolutionary Microbiology* **69(1)**:220–226 DOI 10.1099/ijsem.0.003135.

Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27(21)**:2957–2963 DOI 10.1093/bioinformatics/btr507.

Manter DK, Delgado JA, Holm DG, Stong RA. 2010. Pyrosequencing reveals a highly diverse and cultivar-specific bacterial endophyte community in potato roots. *Microbial Ecology* **60(1)**:157–166 DOI 10.1007/s00248-010-9658-x.

Martins G, Lauga B, Miot-Sertier C, Mercier A, Lonvaud A, Soulas ML, Soulas G, Masneuf-Pomarède I. 2013. Characterization of epiphytic bacterial communities from grapes, leaves, bark and soil of grapevine plant grown, and their relations. *PLOS ONE* **8(8)**:e73013 DOI 10.1371/journal.pone.0073013.

McCalley CK, Woodcroft BJ, Hodgkins SB, Wehr RA, Kim E-H, Mondav R, Crill PM, Chanton JP, Rich VI, Tyson GW, Saleska SR. 2014. Methane dynamics regulated by microbial community response to permafrost thaw. *Nature* **514(7523)**:478–481 DOI 10.1038/nature13798.

Mocali S, Bertelli E, Cello FD, Mengoni A, Sfalanga A, Viliani F, Caciotti A, Tegli S, Surico G, Fani R. 2003. Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. *Research in Microbiology* **154(2)**:105–114 DOI 10.1016/S0923-2508(03)00031-7.

Moore FP, Barac T, Borremans B, Oeyen L, Vangronsveld J, Van Der Lelie D, Campbell CD, Moore ERB. 2006. Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: the characterization of isolates with potential to enhance phytoremediation. *Systematic and Applied Microbiology* **29(7)**:539–556 DOI 10.1016/j.syapm.2005.11.012.

Nakagawa Y, Kawasaki H. 2001. Method for determining the sequence of 16S rRNA gene. In: Miyadoh S, ed. *Identification Manual of Actinomycetes*. Tokyo: Business Center for Academic Societies, 88–117.

Navarrete AA, Kuramae EE, De Hollander M, Pijl AS, Van Veen JA, Tsai SM. 2013. Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiology Ecology* **83(3)**:607–621 DOI 10.1111/1574-6941.12018.

Parte AC. 2018. LPSN-list of prokaryotic names with standing in nomenclature. *Available at* http://www.bacterio.net/index.html (accessed 12 January 2018).

Puspita ID, Kamagata Y, Tanaka M, Asano K, Nakatsu CH. 2012. Are uncultivated bacteria really uncultivable? *Microbes and Environments* **27(4)**:356–366 DOI 10.1264/jsme2.ME12092.

Redford AJ, Bowers RM, Knight R, Linhart Y, Fierer N. 2010. The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environmental Microbiology* **12(11)**:2885–2893 DOI 10.1111/j.1462-2920.2010.02258.x.

Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu W-T, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T. 2013. Insights

into the phylogeny and coding potential of microbial dark matter. *Nature* **499(7459)**:431–437 DOI 10.1038/nature12352.

Roesch LFW, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME Journal* **1(4)**:283–290 DOI 10.1038/ismej.2007.53.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4(4)**:406–425 DOI 10.1093/oxfordjournals.molbev.a040454.

Sakai K. 2001. Chemistry of bark. In: Hon DN, Shiraishi N, eds. *Wood and Cellulosic Chemistry*. Second Edition. New York: Marcel Dekker, 243–273.

Schlaeppi K, Dombrowski N, Oter RG, Van Themaat EVL, Schulze-Lefert P. 2010. Quantitative divergence of the bacterial root microbiota in *Arabidopsis thaliana* relatives. *Proceedings of the National Academy of Sciences of the United States of America* **111(2)**:585–592 DOI 10.1073/pnas.1321597111.

Serkebaeva YM, Kim Y, Liesack W, Dedysh SN. 2013. Pyrosequencing-based assessment of the bacteria diversity in surface and subsurface peat layers of a northern wetland, with focus on poorly studied phyla and candidate divisions. *PLOS ONE* **8(5)**:e63994 DOI 10.1371/journal.pone.0063994.

Shen SY, Fulthorpe R. 2015. Seasonal variation of bacterial endophytes in urban trees. *Frontiers in Microbiology* **6(333)**:427 DOI 10.3389/fmicb.2015.00427.

Stewart EJ. 2012. Growing unculturable bacteria. *Journal of Bacteriology* **194(16)**:4151–4160 DOI 10.1128/JB.00345-12.

Taghavi S, Carafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronaveld J, Van Der Lelie D. 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growing and development of poplar trees. *Applied and Environmental Microbiology* **75(3)**:748–757 DOI 10.1128/AEM.02239-08.

Tahon G, Willems A. 2017. Isolation and characterization of aerobic anoxygenic phototrophs from exposed soils from the Sør Rondane Mountains, East Antarctica. *Systematic and Applied Microbiology* **40(6)**:357–369 DOI 10.1016/j.syapm.2017.05.007.

Tahon G, Tytgat B, Lebbe L, Carlier A, Willems A. 2018. *Abditibacterium utsteinense* sp. nov., the first cultivated member of candidate phylum FBP, isolated from ice-free Antarctic soil samples. *Systematic and Applied Microbiology* **41(4)**:279–290 DOI 10.1016/j.syapm.2018.01.009.

Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y. 2009. Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environmental Microbiology* **11(7)**:1827–1834 DOI 10.1111/j.1462-2920.2009.01907.x.

Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M, Kamagata Y. 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. *Applied and Environmental Microbiology* **71(4)**:2162–2169 DOI 10.1128/AEM.71.4.2162-2169.2005.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10(3)**:512–526 DOI 10.1093/oxfordjournals.molbev.a040023.

Tanaka Y, Matsuzawa H, Tamaki H, Tagawa M, Toyama T, Kamagata Y, Mori K. 2017. Isolation of novel bacteria including rarely cultivated phyla, *Acidobacteria* and *Verrucomicrobia*, from the roots of emergent plants by simple culturing method. *Microbes and Environments* **32(3)**:288–292 DOI 10.1264/jsme2.ME17027.

Tanaka Y, Tamaki H, Matsuzawa H, Nigaya M, Mori K, Kamagata Y. 2012. Microbial community analysis in the roots of aquatic plants and isolation of novel microbes including an organism of the candidate phylum OP10. *Microbes and Environments* **27(2)**:149–157 DOI 10.1264/jsme2.ME11288.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22(22)**:4673–4680 DOI 10.1093/nar/22.22.4673.

Tytgat B, Verleyen E, Sweetlove M, D'hondt S, Clercx P, Van Ranst E, Peeters K, Roberts S, Namsaraev Z, Wilmotte A, Vyverman W, Willems A. 2016. Bacterial community composition in relation to bedrock type and macrobiota in soils from the Sør Rondane Mountains, East Antarctica. *FEMS Microbiology Ecology* **92(9)**:fiw126 DOI 10.1093/femsec/fiw126.

Ulrich K, Ulrich A, Ewald D. 2007. Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiology Ecology* **63(2)**:169–180 DOI 10.1111/j.1574-6941.2007.00419.x.

Vorholt JA. 2012. Microbial life in the phyllosphere. *Nature Reviews Microbiology* **10(12)**:828–840 DOI 10.1038/nrmicro2910.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian classifier for rapid assignment of eRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73(16)**:5261–5267 DOI 10.1128/AEM.00062-07.

Ward NL, Challacombe JF, Janssen PH, Henrissat B, Coutinho PM, Wu M, Xie G, Haft DH, Sait M, Badger J, Barabote RD, Bradley B, Brettin TS, Brinkac LM, Bruce D, Creasy T, Daugherty SC, Davidsen TM, DeBoy RT, Detter JC, Dodson RJ, Durkin AS, Ganapathy A, Gwinn-Giglio M, Han CS, Khouri H, Kiss H, Kothari SP, Madupu R, Nelson KE, Nelson WC, Paulsen I, Penn K, Ren Q, Rosovitz MJ, Selengut JD, Shrivastava S, Sullivan SA, Tapia R, Thompson LS, Watkins KL, Yang Q, Yu C, Zafar N, Zhou L, Kuske CR. 2009. Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. *Applied and Environmental Microbiology* **75(7)**:2046–2056 DOI 10.1128/AEM.02294-08.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173(2)**:697–703 DOI 10.1128/jb.173.2.697-703.1991.

Weiss A, Scheller F, Oggenfuss M, Walsh F, Frey JE, Drissner D, Schmidt H. 2015. Analysis of the bacterial epiphytic microbiota of oak leaf lettuce with 16S ribosomal RNA gene analysis. *Journal of Microbiology, Biotechnology and Food Sciences* **5(3)**:271–276 DOI 10.15414/jmbfs.2015/16.5.3.271-276.

Whitman WB, Coleman DC, Wiebe WJ. 1998. Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences of the United States of America* **95(12)**:6578–6583 DOI 10.1073/pnas.95.12.6578.

Wieland G, Neumann R, Backhaus H. 2001. Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Applied and Environmental Microbiology* **67(12)**:5849–5854 DOI 10.1128/AEM.67.12.5849-5854.2001.

Yamada K, Okuno Y, Meng XY, Tamaki H, Kamagata Y, Hanada S. 2014. *Granulicella cerasi* sp. nov., an acidophilic bacterium isolated from cherry bark. *International Journal of Systematic and Evolutionary Microbiology* **64(Pt 8)**:2781–2785 DOI 10.1099/ijs.0.058636-0.

Zarraonaindia I, Owens SM, Weisenhorn P, West K, Hampton-Marcell J, Lax S, Bokulich NA, Mills DA, Martin G, Taghavi S, Van Der Lelie D, Gilbert A. 2015. The soil microbiome influences grapevine-associated microbiota. *American Society for Microbiology* **6(2)**:e02527-14 DOI 10.1128/mBio.02527-14.