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Diversity of *Methylobacterium* species associated with New Zealand native plants

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Abstract

Methylobacterium species are abundant colonizers of the phyllosphere due to the availability of methanol, a waste product of pectin metabolism during plant cell division. The phyllosphere is an extreme environment, with a landscape that is heterogeneous and continuously changing as the plant grows and is exposed to high levels of ultraviolet irradiation. Geographically, New Zealand (NZ) has been isolated for over a million years, has a biologically diverse flora, and is considered a biodiversity hotspot, with most native plants being endemic. We therefore hypothesize that the phyllosphere of NZ native plants harbor diverse groups of *Methylobacterium* species. Leaf imprinting using methanol-supplemented agar medium was used to isolate bacteria, and diversity was determined using ARDRA and 16S rRNA gene sequencing. *Methylobacterium* species were successfully isolated from the phyllosphere of 18 of the 20 native NZ plant species in this study, and six different species were identified: *M. marchantiae, M. mesophilicum, M. adhaesivum, M. komagatae, M. extorquens*, and *M. phyllosphaerae*. Other α, β, and γ-Proteobacteria, Actinomycetes, Bacteroidetes, and Firmicutes were also isolated, highlighting the presence of other potentially novel methanol utilizers within this ecosystem. This study identified that *Methylobacterium* are abundant members of the NZ phyllosphere, with species diversity and composition dependent on plant species.

Keywords: *Methylobacterium*; phyllosphere; New Zealand; methanol

Introduction

The phyllosphere (above ground parts of plants comprising mainly stems and leaves) is the Earth's biggest biological surface, estimated to be two times bigger than the land surface (Woodward and Lomas 2004). The physicochemical environment of the phyllosphere is highly diverse due to fluctuating nutrient and water availability, temperature, wind pressure, exposure to pollutants, UV radiation, and the variable biology of the waxy protective layer (plant cuticle). This diverse environment provides a habitat for many bacteria (Vorholt 2012), with the planetary phyllosphere bacterial population estimated to be as large as 10^{26} cells (Lindow and Brandl 2003). Global processes of carbon, oxygen, and nitrogen cycling are greatly influenced by phyllosphere bacteria, as is plant health, with growth and productivity being enhanced (Trotsenko et al. 2001, Lindow and Brandl 2003). The number and composition of phyllosphere bacteria are greatly influenced by plant species, sampling site, growing season, plant growth stage, location on the plant, leaf properties, and surrounding plant species (Kinkel et al. 2000, Redford et al. 2010, Finkel et al. 2011).

The genus *Methylobacterium* is a dominant group of *Bacteria* in the phyllosphere (Delmotte et al. 2009) and has been estimated at 10^4–10^7 cells per gram of fresh plant material (Holland et al. 2002). The *Methylobacterium* association with plants can be epiphytic or endophytic (Corpe and Rheem 1989, Delmotte et al. 2009, Knief et al. 2010); or symbiotic (Sy et al. 2001); however, there are no reports of *Methylobacterium* as the cause of plant disease. The association of *Methylobacterium* spp. with plants typically relies on methanol, a volatile organic compound (VOC), which is released by plants during growth through stomatal pores in the epidermis (Galbally and Kirstine 2002). However, other plant-derived carbon compounds may also support their colonization (Sy et al. 2005, Delmotte et al. 2009). Methanol is produced inside leaves as a byproduct of pectin metabolism during cell wall synthesis. During cell elongation and division pectin methylesterases catalyze the C-6 demethylation of homogalacturonan within plant cell walls, as a result, methanol is released (Korner et al. 2009). The global methanol emission from plants is estimated to be 100–128 Tg per year (Galbally and Kirstine 2002). Colonization of plants by methylotrophic bacteria, especially *Methylobacterium* species, is of interest because they play an important role in the atmospheric methanol cycle by utilizing methanol as their sole source of carbon and energy (Corpe and Rheem 1989). They also produce plant growth-promoting substances such as cytokinins, auxins, and vitamin B_{12} (Trotsenko et al. 2001, Ivanova et al. 2006) and are involved in seed germination, root development, and increased yield of agricultural plants (Meena et al. 2012).

Members of the genus *Methylobacterium* are pink-pigmented facultative methylotrophs (PPFMs) (Kelly et al. 2014). They utilize one-carbon compounds, including methanol (CH_3OH), methylamine (CH_3NH_2), and formaldehyde (CH_2O), and multi-carbon compounds containing no carbon–carbon bonds, as well as organic substrates with carbon–carbon bonds as the sole source of carbon and energy (Kelly et al. 2014). They are strict aerobes belonging to the α-*Proteobacteria*, order *Rhizobiales*, and are Gram-negative and rod-shaped organisms. *Methylobacterium* are found worldwide on the leaves of many different plant species, and

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studies have shown that *Methylobacterium* species composition varies within and between plant species (Balachandar et al. 2008, Knief et al. 2008). However, geographical location has been shown to have a stronger influence than plant species on both *Methylobacterium* and phyllosphere community composition (Knief et al. 2010, Wellner et al. 2011).

The objective of this study was to investigate the diversity of *Methylobacterium* species in the phyllosphere of native New Zealand (NZ) plants, via the isolation of methanol-utilizing bacteria. Amplified ribosomal DNA restriction analysis (ARDRA) was used to select representative isolates for sequencing. This study is significant as the first exploration of the species composition of the genus *Methylobacterium* in the phyllosphere of native NZ plants.

Materials and methods

Sample collection and bacterial isolation

Twenty different native NZ plants were selected for isolation of *Methylobacterium* species (Table 1), plants were chosen to represent a diversity of plant types, including trees, shrubs, herbs, ferns, and flax. Leaves (three) from five plants of each plant species were collected in sterile containers, with the majority collected from the campus of the University of Waikato (Hamilton, NZ), where they grow naturally. Leaves collected were healthy but not new growth, and were all collected in the morning. Leaf-imprinting was used to isolate bacteria from the leaf surface (Corpe 1985). Immediately after collection, leaves were laid directly on the surface of 0.5% methanol-supplemented ammonium mineral salt (AMS) agar plates and impressed carefully, large leaves were cut to the desired size and small leaves used whole, plates were then sealed with parafilm, and incubated at 30°C for up to two weeks (Holland and Polacco 1994). AMS agar medium contained: 0.7 g/l K_2HPO_4, 0.54 g/l NH_4Cl, 0.1 mg/l $ZnSO_4.7H_2O$, 0.03 mg/l $MnCl_2.4H_2O$, 0.3 mg/l H_3BO_3, 0.2 mg/l $CoCl_2.6H_2O$, 0.01 mg/l $CuCl_2.2H_2O$, 0.02 mg/l $NiCl_2.6H_2O$, 0.06 mg/l $Na_2MoO_4.2H_2O$, 15.0 g/l Difco™ agar (1.5%), in distilled water, pH was adjusted to 6.8 and the medium sterilized by autoclaving (Whittenbury et al. 1970). An amount of 0.5% methanol was added aseptically to the autoclaved medium upon cooling and mixed thoroughly before the plates were poured. After incubation, colonies were chosen randomly from the plates and streaked. Colonies were re-streaked 5–6 times on fresh AMS agar plates to obtain a pure culture.

DNA extraction from pure cultures

For ARDRA analysis, DNA was extracted from bacterial colonies picked with a sterile toothpick into a 1.5-ml microfuge tube containing 1 ml of sterile H_2O. The tube was vortexed vigorously until cells were dispersed, boiled for 10 min, centrifuged for 5 min at 1000 rpm, then kept on ice. The supernatant (5 μl) was used as a PCR template.

Prior to the extraction of DNA for sequencing (Marmur 1961), isolates were grown in 50 ml AMS plus methanol broth at 30°C, transferred into 50 ml sterile falcon tubes, and centrifuged for 3 min at 3000 rpm. The supernatant was then removed and the cell pellet resuspended in 400 μl SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris.HCl). Lysozyme solution (50 mg/ml in TE plus 10 mM NaCl) was added (20 μl) and incubated at 37°C for 1 hour. After incubation, 20 μl of 20% SDS and 10 μl of proteinase K solution (20 mg/ml in TE) were added to the lysates and incubated at 60°C for 3 hours. The digested lysates were then purified by phenol chloroform extraction, concentrated by ethanol precipitation, and DNA was stored at −20°C. DNA concentration was quantified using a NanoDrop™ 1000 spectrophotometer and concentration was adjusted to 50–60 ng/μl with TE buffer.

PCR amplification and ARDRA of 16S rRNA genes

The 16S rRNA gene was amplified using universal eubacterial primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). For each isolate, 5 μl of template DNA was added to 45 μl PCR master mix in 200 μl thin-walled PCR tubes. Master mix for each reaction contained 24.75 μl of sterile water, 5 μl of 10x PCR buffer, 5 μl of $MgCl_2$ (50 mM), 5 μl of 2 mM dNTPs, 2 μl of each primer (10 μM) (Integrated DNA Technologies, Inc), 1 μl of bovine serum albumin (Promega Corporation, USA), and 0.25 μl of *Taq* DNA polymerase (Invitrogen, NZ). PCR was run on a Bio-Rad DNA Engine® (PTC-200) Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA, USA). The thermal cycling conditions consisted of 35 cycles (30 sec at 94°C 30 sec annealing at 60°C, and 1 min 30 sec at 72°C) with an initial denaturation of 2 min at 94°C, and a final extension step of 10 min at 72°C. PCR products were visualized by gel electrophoresis, and product size estimated using a 250-bp DNA ladder (Invitrogen).

ARDRA is an established method for determining taxonomic relatedness between isolates (Fisher and Triplett 1999), which were screened to select representatives for sequencing. The 16S rRNA gene PCR amplicon of each isolate was digested with Rsa I, and the products were analyzed by gel electrophoresis using 2% agarose in 1X TAE buffer. A 1 kb + DNA marker (Invitrogen) was run on every gel to size the restriction fragments. All isolates were compared visually for matching fingerprints and grouped into different restriction types. Representative isolates from each restriction group were selected randomly for sequencing.

16S rRNA gene sequencing and analysis

PCR products of isolates selected for sequencing were purified using ExoSAP (Affymetrix, Ohio, USA), according to the manufacturer's protocol. Sequencing reactions were performed at the Waikato DNA Sequencing Facility with primers 27F and 1492R on an ABI 3130XL (Applied Biosystems). Each 16S rRNA gene sequence was analyzed for closest identities using BLASTN in the NCBI database (Altschul et al. 1990).

Results

To investigate *Methylobacterium* species diversity on NZ native plants, a total of 20 plant species from different plant types (tree, shrub, herb, fern, and flax) were selected. After 10–14 days incubation of leaf-imprinted agar plates at 30°C, colonies were selected for streaking on fresh plates, and over an extended period, these were re-streaked 4–5 times, resulting in the isolation of 245 pure cultures of methanol-grown strains. The majority of isolates (83%) were pale to vivid pink pigmented, with the remaining isolates either cream (10%) or dark orange to red (7%). Methylotrophs were isolated from the leaves of every plant species, but the number isolated varied between species (Table 2). Most isolates were from *Micropiper excelsum* (21.6%), followed by *P. regius* (20%), *P. tenax* (18.8%), *P. cookianum* (11.4%), and *A. oblongifolium* (7.3%), with the lowest numbers from *B. novea-zealandiae*, *Cyathea dealbata*, *C. medullaris*, and *K. excelsa* (0.4%).

The 16S rRNA gene was amplified from all isolates and analyzed by ARDRA, which resulted in a number of different restriction patterns, reflecting the diversity of methylotrophs isolated.

Table 1. NZ native plant species used in this study.

Scientific name	Common/Maori name	Family
Agathis australis	Kauri	*Araucariaceae*
Alectryon excelsus	Titoki	*Sapindaceae*
Asplenium oblongifolium	Shining Spleenwort/Huruhuruwhenua	*Aspleniaceae*
Blechnum novea-zealandiae	Palm-leaf fern/Kiokio	*Blechnaceae*
Coprosma robusta	Karamu	*Rubiaceae*
Cyathea cunninghamii	Gully tree fern	*Cyatheaceae*
Cyathea dealbata	Silver tree fern/Ponga	*Cyatheaceae*
Cyathea medullaris	Black tree fern/Mamaku	*Cyatheaceae*
Griselinia littoralis	Kapuka	*Griseliniaceae*
Hebe elliptica	Kokomuka	*Plantaginaceae*
Knightia excelsa	Rewarewa	*Proteaceae*
Macropiper excelsum	Kawakawa	*Piperaceae*
Melicytus ramiflorus	Mahoe	*Violaceae*
Metrosideros excelsa	Pohutokawa	*Myrtaceae*
Olearia traversii	Chatham Island Akeake	*Asteraceae*
Olearia albida	Tree daisy/Tanguru	*Asteraceae*
Phormium cookianum	Mountain Flax/Wharariki	*Hemerocallidaceae*
Phormium tenax	Common Flax/Harakeke	*Hemerocallidaceae*
Pittosporum tenuifolium	Black Matipo/Kohuhu	*Pittosporaceae*
Plagianthus regius	Ribbonwood/Manatu	*Malvaceae*

Table 2. Isolates from each plant species grouped by ARDRA analysis.

	Number of isolates in each OTU																
Plant species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Total**
Agathis australis		3															3
Alectryon excelsus	3	2	1						1								7
Asplenium oblongifolium	9	3	1		1							1				3	18
Blechnum novea-zealandiae		1															1
Coprosma robusta		2															2
Cyathea cunninghamii	2	1															3
Cyathea dealbata			1														1
Cyathea medullaris			1														1
Griselinia littoralis		4	1														5
Hebe elliptica	1	2															3
Knightia excelsa		1															1
Macropiper excelsum	21	13	8	1										1	2	7	53
Melicystus ramiflorus	1	1	2							1							5
Metrosideros excelsa		5															5
Olearia traversii		3	2														5
Olearia albida	3								1	1							5
Phormium cookianum		13	7		3				1	1						3	28
Phormium tenax	7	27	1				1	2	2	1	1		2			2	46
Pittosporum tenuifolium	3	1															4
Plagianthus regius	17	25	1	1	1	1				1			2				49
Total	67	107	26	2	5	1	1	2	5	5	1	1	4	1	2	15	245

Isolates with identical restriction patterns were grouped into 16 operational taxonomic units (OTUs) (Table 2). The majority of isolates (87.7%) grouped in four OTUs: OTU 2 with 107 isolates (43.7%), OTU 1 (67 isolates, 27.3%), OTU 3 (26 isolates, 10.6%), and OTU 16 (15 isolates, 6.1%). OTUs 5, 9, and 10 each had 5 isolates (2.0%), followed by OTU 13 (4 isolates, 1.6%), OTU 4, 8, and 15 (2 isolates each, 0.8%), and the remaining OTUs each had only one isolate (0.4%).

From the ARDRA analysis, a total of 31 isolates were selected randomly as representatives from each OTU for sequencing. BLAST analysis of the 16S rRNA gene sequences (Table 3) revealed that most isolates (79.6%, 195 of 245) were members of the genus *Methylobacterium*, the majority (54.9%, 107 of 195) belonged to OTU 2 and had 98%–99% identity to *M. mesophilicum* or *M. komagatae*; followed by OTU 1 (34.4%, 67 of 195) with 98%–99% identity to *M. marchantiae* or *M. adhaesivum*; OTU 16 (7.7%, 15) with 97%–99% identity to *M. phyllosphaerae*; OTU 13 (2.1%, 4) with 99% identity to *M. extorquens*; and OTU 4 (1.0%, 2) with 99% identity to *M. marchantiae*. Other α-*Proteobacteria* methylotrophs isolated included 28 (11.4% of total isolates) members of the genus *Hyphomicrobium*, closely related (98%–99%) to *H. facile* (OTU 3 and 15), five isolates (OTU 10) related to *Methylopila capsulata* (86%), and one isolate (OTU 14) related to *Rhizobium endophyticum* (95%). β-*Proteobacteria* isolates included five (OTU 5) closely related to *Methylophilus methylotrophus* (99%), one isolate (OTU 6) closely related to *Ramlibacter ginsenosidimutans* (99%), and one isolate (OTU

Table 3. BLAST analysis of 16S rRNA gene sequences of isolates from each OTU.

OTU	Isolates (%)	Isolate	Isolation source	BLAST match	ID (%)	Matched bp
***Methylobacterium* isolates**						
1	27.3	kk002	*Macropiper excelsum*	*Methylobacterium marchantiae* AB698705	99	993/1005
		kk040			99	974/988
		rw104	*Plagianthus regius*		99	753/756
		kh130	*Pittosporum tenuifolium*		99	812/824
		cf283	*Phormium tenax*	*Methylobacterium adhaesivum* KF681060	98	656/671
2	43.7	kk034	*Micropiper excelsum*	*Methylobacterium komagatae* AB703238	98	647/666
		kk036		*Methylobacterium mesophilicum* KF573002	99	829/838
		rw080	*Plagianthus regius*		99	316/320
		pk208	*Metrosideros excelsa*		98	809/822
		ss136	*Asplenium oblongifolium*		99	641/649
4	0.8	rw087	*Plagianthus regius*	*Methylobacterium marchantiae* AB698705	99	672/680
13	1.6	rw086	*Plagianthus regius*	*Methylobacterium extorquens* LT962688	99	639/646
16	6.1	kk037	*Micropiper excelsum*	*Methylobacterium phyllosphaerae* CP015367	99	713/723
		kk048			99	606/609
		pk243	*Metrosideros excelsa*		97	586/605
		cf284	*Phormium tenax*		99	934/942
Other Methylotrophic isolates						
3	10.6	kk004	*Micropiper excelsum*	*Hyphomicrobium facile* MG846099	99	801/813
		kk063			99	730/739
		ss288	*Asplenium oblongifolium*		99	699/709
5	2.0	rw113	*Plagianthus regius*	*Methylophilus methylotrophus* LC191544	99	703/707
6	0.4	rw083	*Plagianthus regius*	*Ramlibacter ginsenosidimutans* KY649387	99	476/481
7	0.4	cf159	*Phormium tenax*	*Alcaligenes faecalis* DQ379508	97	275/283
8	0.8	cf150	*Phormium tenax*	*Janibacter melonis* JN084150	94	472/502
9	2.0	cia197	*Olearia traversii*	*Niastella populi* AB682649	99	666/675
		ti234	*Alectryon excelsus*			
10	2.0	cia203	*Olearia traversii*	*Methylopila capsulata* AJ634928	86	258/299
11	0.4	cf153	*Phormium tenax*	*Paenibacillus lautus* LT601284	99	676/685
12	0.4	ss132	*Asplenium oblongifolium*	*Xanthomonas translucens* DQ424867	85	584/684
14	0.4	kk035	*Micropiper excelsum*	*Rhizobium endophyticum* NR116477	95	761/803
15	0.8	kk005	*Micropiper excelsum*	*Hyphomicrobium facile* MG846099	98	827/840
		kk022			99	814/824

7) related to *Alcaligenes faecalis* (97%). Of the remaining isolates, one isolate (OTU 12) was a γ-*Proteobacteria* related to *Xanthomonas translucens* (85%), three *Actinobacteria* isolates (OTU 8) related to *Janibacter melonis* (94%), five *Bacteroidetes* isolates (OTU 9) closely related to *Niastella populi* (99%), and one *Firmicutes* isolate (OTU 11) had 99% identity to *Paenibacillus lautus*.

Most plant species harbored methylobacterial communities of differing complexity (Table 2 and Fig. 1), except the tree ferns *C. dealbata* and *C. medullaris*, from which only *Hyphomicrobium* sp. were isolated. The majority of the *Methylobacterium* isolates, 54.9% (OTU 2), were closely related to *M. mesophilicum* or *M. komogatae*, and they were broadly distributed (17 plant species) and common isolates from most plants. Most isolates were from *P. tenax* (25.2%), *P. regius* (23.4%), *M. excelsum* and *P. cookianum* (12.1% each), *M. excelsa* (4.7%), and *G. littoralis* (3.7%). The second-largest group of *Methylobacterium* isolates (34.4%, OTU 1) was closely related to *M. marchantiae* or *M. adhaesivum* and was isolated from ten plant species. Most isolates were from *M. excelsum* (31.3%), *P. regius* (25.4%), *A. oblongifolium* (13.4%), and *P. tenax* (10.4%). The third group of *Methylobacterium* isolates (7.7%, OTU 16) was closely related to *M. phyllosphaerae* but were only isolated from four plant species, namely *A. oblongifolium*, *M. excelsum*, *P. cookianum*, and *P. tenax*, with most isolates (46.7%) from *M. excelsum*. The fourth group (OTU 13) was closely related to *M. extorquens* and was only isolated from two species of plants, *P. tenax* and *P. regius*. The fifth group (OTU 4) was also related to *M. marchantiae* and was only isolated from *M. excelsum* and *P. regius*. No single plant species was found to harbor all of the *Methylobacterium* sp. identified; however, maximum association (with four of the five OTUs) was found with *M. excelsum*, *P. tenax*, and *P. regius*, followed by *A. oblongifolium* (with three of the five OTUs).

Discussion

Methylobacterium species diversity on the leaves of native NZ plants was investigated using leaf imprinting on methanol-supplemented AMS agar. Methanol is a key substrate for the growth of *Methylobacterium*, and in plants, methanol is produced during plant cell division as the waste product of pectin metabolism (Galbally and Kirstine 2002). A total of 245 methylotrophs were isolated from the leaves of 20 different native NZ plants, but the bacterial species and number of isolates varied between plants. Interspecies variability is frequently seen in other studies of the phyllosphere community (Yang et al. 2001, Lambais et al. 2006, Whipps et al. 2008, Redford et al. 2010). In this study, isolates were selected for sequencing using ARDRA, which identified sixteen different groups of isolates. Sequencing of representative isolates from each restriction group revealed that the majority of isolates (79.5%) were *Methylobacterium* species commonly found to colonize plants (Delmotte et al. 2009, Wellner et al. 2011, Knief et al. 2012a, Knief et al. 2012b). Sixteen different phyllosphere *Methylobacterium* species have been identified (Balachandar et al. 2008, Knief et al. 2008), with *M. extorquens* being a ubiquitous colonizer of the phyllosphere of many plants (Del-

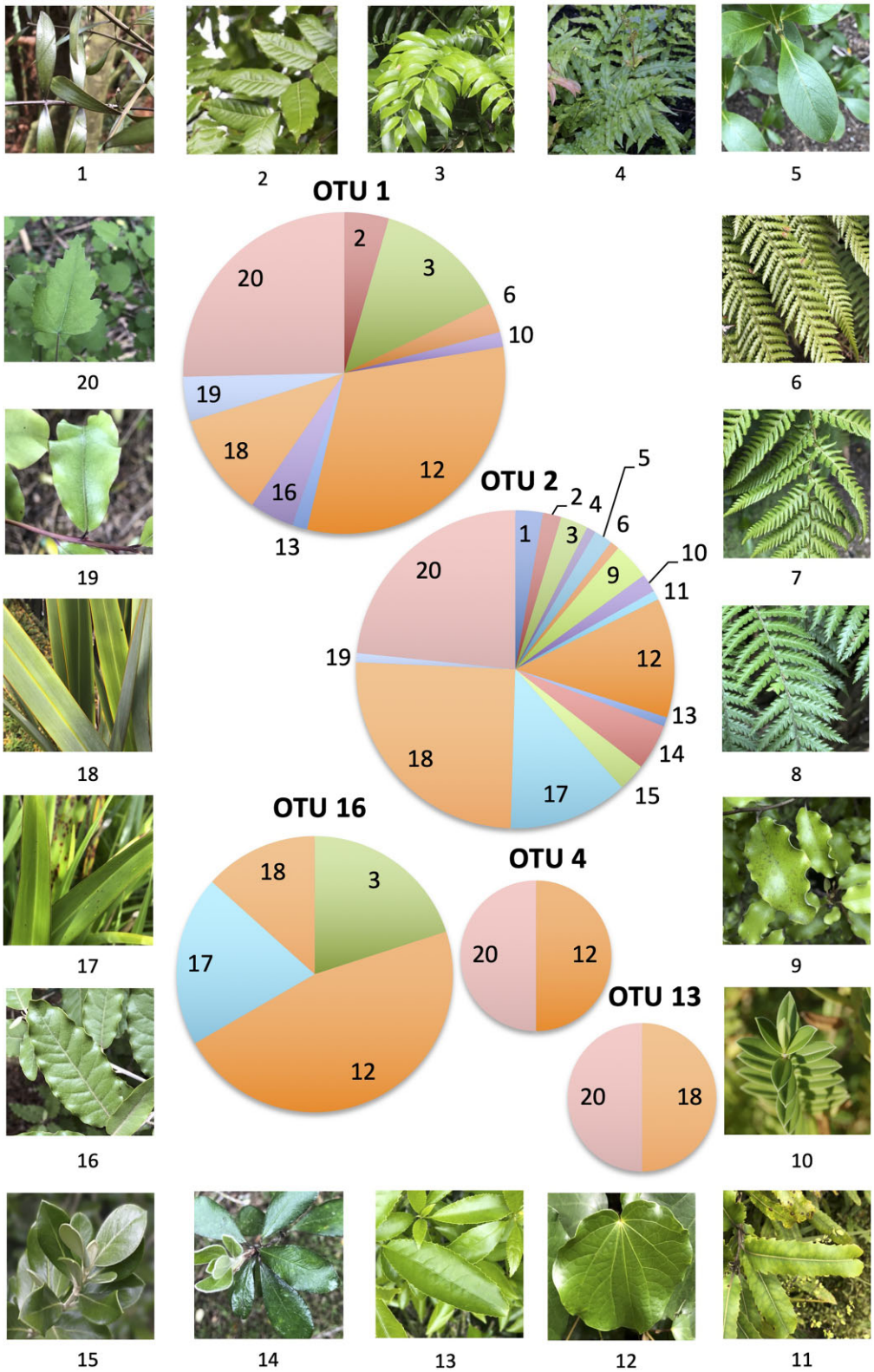

Figure 1. Distribution of *Methylobacterium* isolates from native NZ plants. Pie charts show the number of isolates in each OTU from each plant species, and images show their leaves. The plant species are: 1. *A. australis*, 2. *A. excelsus*, 3. *A. oblongifolium*, 4. *B. novea-zealandiae*, 5. *C. robusta*, 6. *C. cunninghamii*, 7. *C. dealbata*, 8. *C. medullaris*, 9. *G. littoralis*, 10. *Hebe elliptica*, 11. *K. excelsa*, 12. *M. excelsum*, 13. *M. ramiflorus*, 14. *M. excelsa*, 15. *O. traversii*, 16. *Olearia albida*, 17. *P. cookianum*, 18. *P. tenax*, 19. *P. tenuifolium*, 20. *P. regius*.

motte et al. 2009). This study identified six different *Methylobacterium* species isolated from the leaves of native NZ plants. This low number of species may be because the isolation medium used was highly selective, with only methanol as a carbon source, and *Methylobacterium* species are commonly differentiated according to their carbon utilization ability (Kelly et al. 2014). Another reason could be the degree of association between NZ plants and *Methylobacterium* sp., with other studies showing that *Methylobacterium* association can be epiphytic (Omer et al. 2004), endophytic (Lacava et al. 2004), or symbiotic (Jourand et al. 2004). The *Methylobacterium* species identified in this study (*M. marchantiae*, *M. adhaesivum*, *M. komogatae*, *M. mesophilicum*, *M. extorquens*, and *M. phyllosphaerae*) are common leaf epiphytes (Knief et al. 2008, Verginer et al. 2010, Schauer et al. 2011, Tani et al. 2012), not surprisingly given that leaf imprinting was used to isolate the bacteria. The final reason could be geographic location, which has been shown to be an important determinant in shaping *Methylobacterium* colonization of the phyllosphere (Knief et al. 2010); however, this could not be addressed in this study.

Another factor to be considered was the effect of leaf texture and structure on the ease of the isolation of bacteria from different plants. The leaves of plants that yielded the most isolates (*P. regius, M. excelsum*, *P. tenax*, *P. cookianum*, and *A. oblongifolium*) are thin and easily lie on the medium (see Fig. 1), making imprinting easier than for other plants in this study that have leaves that are thick, glossy, hard, shiny, spiky, or velvety (*A. australis*, *A. excelsus*, *C. robusta*, *G. littoralis*, *H. elliptica*, *K. excelsa*, *M. excelsa*, *O. albida*, *O. traversii*, and *P. tenuifolium*). Leaf texture and structure have been shown to be important factors in obtaining epiphytes from the leaf surface using imprinting (Holland et al. 2000). The low number of *Methylobacterium* isolates from *B. novea-zealandiae* and *C. cunninghamii* may have been due to the fact that only small, young plants were sampled. But generally, in this study, few *Methylobacterium* species were isolated from ferns, and none were isolated from *C. dealbata* (black fern) or *C. medullaris* (silver fern), which may indicate that either leaf structure, leaf age (fern leaves have a shorter life span than other NZ native plants), or leaf chemistry which may restrict colonization by methylobacteria. However, plant species have been shown to be the main driver for the community structure of *Methylobacterium* species in several studies (Kinkel et al. 2000, Omer et al. 2004, Knief et al. 2008, Knief et al. 2010, Redford et al. 2010, Wellner et al. 2011) and may therefore be significant for NZ native plant species.

The most frequently isolated *Methylobacterium* colonizer was *M. mesophilicum,* which was isolated from 17 plant species and was the only *Methylobacterium* sp. to be isolated from seven plant species (*A. australis*, *B. novea-zealandiae*, *C. robusta*, *G. littoralis*, *K. excelsa*, *M. excelsa*, and *O. traversii*), possibly indicating some specialization. Similarly, only *M. marchantiae* was isolated from *O. albida*. From this study, it is difficult to explain the reasons for the association of individual *Methylobacterium* sp. with specific plants. However, plant species and the generalist nature of some *Methylobacterium* species have been found to play a combined role in colonization (Dourado et al. 2012).

Other α-Proteobacteria (*Hyphomicrobium*, *Methylopila*, and *Rhizobium*), β-Proteobacteria (*Alcaligenes*, *Methylophilus*, and *Ramlibacter*), γ-Proteobacteria (*Xanthomonas*), Actinomycetes (*Janibacter*), Bacteroidetes (*Niastella*), and Firmicutes (*Paenibacillus*) were also isolated, with similar genera seen in culture-independent studies of the phyllosphere (Jackson et al. 2006, Lambais et al. 2006, Redford et al. 2010, Rastogi et al. 2012). Several studies have demonstrated the association of some of these *Bacteria* with plants, including *Hyphomicrobium* sp. from the *Arabidopsis* phyllosphere (Reisberg et al. 2013), *Methylopila sp.* from banana fruit (Doronina et al. 2013), *R. endophyticum* from the green bean (Lopez-Lopez et al. 2010), and *A. faecalis* from green ash leaves (Sandhu et al. 2009). *Methylophilus* has been found in the phyllosphere (Wellner et al. 2011), *Xanthomonas* sp. have been identified on plant leaves and stems (Corpe and Rheem 1989, Sheng et al. 2011), *Janibacter* have been detected in the rhizosphere (Guiñazú et al. 2013), *N. populi* from the soil of a Euphrates Poplar forest (Zhang et al. 2010), and *P. lautus* from the rhizosphere of wild grass (Sharma et al. 2010). While a number of these genera are known methanol utilizers (*Methylophila*, *Methylophilus*, and *Hypomicrobium*), to date there is no clear evidence for methanol utilization by most of the other genera of *Bacteria* (*Alcaligenes*, *Janibacter*, *Niastella*, *Ramlibacter*, *Rhizobium*, *Paenibacillus*, and *Xanthomonas*), although they did represent only a small number of all the isolates in this study. Further studies are therefore required to understand more about the utilization of carbon substrates by these isolates.

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