

Rhizobia with 16S rRNA and *nifH* Similar to *Mesorhizobium huakuii* but Novel *recA*, *glnII*, *nodA* and *nodC* Genes Are Symbionts of New Zealand *Carmichaelinae*

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Abstract

New Zealand became geographically isolated about 80 million years ago and this separation gave rise to a unique native flora including four genera of legume, *Carmichaelia*, *Clanthus* and *Montigena* in the *Carmichaelinae* clade, tribe Galegeae, and *Sophora*, tribe Sophoreae, sub-family Papilionoideae. Ten bacterial strains isolated from NZ *Carmichaelinae* growing in natural ecosystems grouped close to the *Mesorhizobium huakuii* type strain in relation to their 16S rRNA and *nifH* gene sequences. However, the ten strains separated into four groups on the basis of their *recA* and *glnII* sequences: all groups were clearly distinct from all *Mesorhizobium* type strains. The ten strains separated into two groups on the basis of their *nodA* sequences but grouped closely together in relation to *nodC* sequences; all *nodA* and *nodC* sequences were novel. Seven strains selected and the *M. huakuii* type strain (isolated from *Astragalus sinicus*) produced functional nodules on *Carmichaelia* spp., *Clanthus puniceus* and *A. sinicus* but did not nodulate two *Sophora* species. We conclude that rhizobia closely related to *M. huakuii* on the basis of 16S rRNA and *nifH* gene sequences, but with variable *recA* and *glnII* genes and novel *nodA* and *nodC* genes, are common symbionts of NZ *Carmichaelinae*.

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Introduction

Members of the Fabaceae (the legume family) are components of most of the world's vegetation types [1]. Many legume species have the capacity to fix atmospheric nitrogen (N₂) via symbiotic bacteria (generally termed 'rhizobia') in root nodules and this can give them an advantage under low soil N conditions if other factors are suitable for growth [2,3,4]. New Zealand (NZ) became geographically isolated about 80 million years ago [5,6] and this separation gave rise to a unique native flora [7] that is considered to have evolved during the late Cenozoic [8,9]. There are four genera of legume, of the sub-family Papilionoideae on the main New Zealand islands, plus the strand species *Canavalia rosea* which occurs in the Kermadec Islands [10]. The four genera are the closely related *Carmichaelia* (23 endemic species), *Clanthus* (2 endemic species) and *Montigena* (1 endemic species) in the *Carmichaelinae* clade, tribe Galegeae, and *Sophora* (8 endemic species), within the tribe Sophoreae [11,12,13,14,15]. All species in all four genera are capable of nodulation, but genotypic data on the rhizobia which induce nodules on these plants are limited [4,16].

Previously, sequences were obtained for the small subunit ribosomal RNA (16S rRNA) gene of twenty bacterial strains isolated from species of the four NZ native legume genera growing in natural ecosystems [16]. Most isolates aligned with the genus *Mesorhizobium* either as named species or as putative novel species. Five strains from *Carmichaelia*, and one each from *Clanthus* and *Montigena*, were most closely related to *Mesorhizobium huakuii* (*M. huakuii*). However, this study did not assess the ability of the isolated rhizobial strains to nodulate NZ native legumes or sequence any of their N₂-fixation (*nif*) or nodulation (*nod*) genes. A separate study, characterised two bacterial strains, Ca004 and Cc3, isolated from *Carmichaelia australis* and *Carmichaelia corrugata* respectively, growing in natural ecosystems, using 16S rRNA sequences [17]. Both strains aligned closely with *M. huakuii* and produced functional nodules on the five *Carmichaelia* spp. tested. In the current study, six strains isolated from *Carmichaelia* spp. and *Montigena* aligned with *M. huakuii* on the basis of their 16S rRNA sequences.

Here we focus on bacterial strains isolated from NZ native legumes growing in natural ecosystems in the current and previous studies which aligned closely with *M. huakuii* on the basis of their

16S rRNA sequences. In addition to 16S rRNA, selected 'housekeeping' and *nif* and *nod* genes were sequenced from the strains and their ability, and that of the *M. huakuii* type strain originally isolated from *Astragalus sinicus* [18,19], to nodulate species of the different NZ native legume genera and *A. sinicus* is assessed.

Materials and Methods

Bacterial strains and culture media

Ten bacterial strains isolated from species in the *Carmichaelinae* growing in natural ecosystems in the current and previous studies were examined. All strains are deposited in the International Collection of Microorganisms from Plants (ICMP), Landcare Research, Auckland, NZ. Strains ICMP 18942, ICMP 18943 and ICMP 19420 from *Montigena novae-zelandiae* (Mn) and ICMP 19041, ICMP 19042 and ICMP 19043 from *C. australis* (Ca), *C. monroi* (Cm) and *C. nana* (Cn) respectively were isolated in the current study from plants sampled at Dry Stream, Torlesse Range, Canterbury, NZ (43°16'S 171°43'E) in April 2010. All necessary permits for the described field studies were obtained from the Department of Conservation, NZ. Christine Fernyhough and John Bougen, owners of Castle Hill Station, are thanked for permission to collect plants, seed and rhizobia from Dry Stream. Strains ICMP 11541, ICMP 12690 and ICMP 13190 isolated previously from *Clanthus puniceus* (Clp), *Montigena novae-zelandiae* and *C. australis* respectively [16] and the *M. huakuii* type strain (ICMP 11069) were obtained from the ICMP collection directly. Strain ICMP 19418 (= strain Ca004) [17] was sourced from the University of York rhizobium collection.

For strains isolated in the current study, root nodules were surface sterilised in a laminar flow cabinet by immersion in 96% ethanol for 5 seconds and 5% sodium hypochlorite for 3 minutes then rinsed with sterile water. Surface sterilised nodules were squashed in sterile water then this suspension was streaked onto a yeast mannitol agar (YMA) [20] plate and incubated at 20–25°C in the dark for 2–4 days. A purified culture or a single colony by sub-culture was obtained from each plate. Samples of these cultures and those from the ICMP collection were inoculated into a suspension of Yeast Mannitol Broth (YMB) [20] and used for preparation of subcultures for DNA extraction or inoculum.

Sequencing of the 16S rRNA, housekeeping and symbiotic genes

DNA was extracted from the bacterial cultures using the standard Qiagen-Gentra PUREGENE DNA Purification Kit for gram-negative bacteria. Six genes were studied: 16S rRNA, DNA recombinase A (*recA*), glutamine synthetase II (*glnII*), nitrogenase iron protein (*nifH*), N-acetyltransferase nodulation protein A (*nodA*) and N-acetylglucosaminyl transferase nodulation protein C (*nodC*). Primers for PCR amplification with their sequences and sources are shown in Table 1. All primers were manufactured by Integrated DNA Technologies, Auckland, NZ. All PCR amplifications were performed using the FastStart™ Taq DNA Polymerase kit (Roche Applied Science, Auckland) optimised for annealing temperature and primer concentration, if required. The PCR products were resolved via gel electrophoresis (1% agarose gel in 1×Tris-acetate-EDTA buffer) followed by staining with ethidium bromide solution and viewing under UV light. PCR products were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University, Lincoln, NZ and DNA sequence data were obtained via Sequence Scanner v 1.0 software (©Applied Biosystems) and edited and assembled using DNA-MAN Version 6 (©Lynnon Biosoft Corporation).

Table 1. Oligonucleotide primers used in this study.

Target gene	Primer	Sequence (5'-3')*	Reference
16S rRNA	F27	AGA-GTT-TGA-TCM-TGG-CTC-AG	[21]
	FGPS485F	CAG-CAG-CCG-CGG-TAA	[22]
	R1494	CTA-CGG-YTA-CCT-TGT-TAC-GAC	[21]
<i>recA</i>	41F	TTC-GGC-AAG-GGM-TCG-RTS-ATG	[23]
	640R	ACA-TSA-CRC-CGA-TCT-TCA-TGC	
<i>glnII</i>	GSII-1	AAC-GCA-GAT-CAA-GGA-ATT-CG	[24]
	GSII-2	ATG-CCC-GAG-CCG-TTC-CAG-TC	
	GSII-3	AGR-TYT-TCG-GCA-AGG-GYT-C	
	GSII-4	GCG-AAC-GAT-CTG-GTA-GGG-GT	
<i>nifH</i>	PolF	TGC-GAY-CCS-AAR-GCB-GAC-TC	[25]
	PolR	ATS-GCC-ATC-ATY-TCR-CCG-GA	
<i>nodC</i>	α-nodCF	AYG-THG-TYG-AYG-ACG-GTT-C	[26]
	α-nodCR	CGY-GAC-AGC-CAN-TCK-CTA-TTG	
<i>nodA</i>	nodA1	TGC-RGT-GGA-ARN-TRN-NCT-GGG-AAA	[27]
	nodA3	TCA-TAG-CTC-YGR-ACC-GTT-CCG	[28]

*A, C, G, T = standard nucleotides; M = C or A; Y = C or T; R = A or G; S = G or C; B = T or C or G; H = A or C or T; N = A or G or C or T; K = T or G.
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Phylogenetic analyses

DNA sequences were aligned and Maximum Likelihood trees constructed with 500 bootstrap replications with partial deletion and an 80% coverage cut off using MEGA5 software [29]. The most closely related *Mesorhizobium* type strains and strains isolated from NZ native legumes which were closely related to *M. huakuii* on the basis of 16S rRNA and available on the Genbank sequence database (www.ncbi.nlm.nih.gov/genbank) were used for the 16S rRNA, *recA*, *glnII*, *nifH*, *nodA* and *nodC* trees. For the *nodA* tree, we also included the sequence from a strain (TM1) recently isolated from *Thermopsis lupinoides* and characterised as *M. huakuii* on the basis of 16S rRNA [30]. For the *nodC* tree, strains characterised as *M. huakuii* on the basis of their 16S rRNA sequences were used as there is no *nodC* sequence for the *M. huakuii* type strain in Genbank and we were unable to sequence it. MEGA5 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian Information Criterion (BIC) score) used for each gene. The Kimura 2-parameter (K2), gamma distribution (+G) with invariant sites (+I) model was used for 16S rRNA. The Tamura 3-parameter (T92), gamma distribution (+G) model was used for all other genes. Only bootstrap probability values ≥50% are shown. The sequences obtained in this study have been deposited in the GenBank sequence database and their accession numbers are shown in the figures.

Nodulation and N₂ fixation studies

Seven bacterial strains (ICMP 12690 (Mn), ICMP 18942 (Mn), ICMP 13190 (Ca), ICMP 19041 (Ca), ICMP 19042 (Cm), ICMP 19418 (Ca) and ICMP 11541 (Clp)) were selected for nodulation and N₂ fixation studies. Seeds of *Carmichaelia* spp. and *M. novae-zelandiae* were collected from the field site (Dry Stream, Torlesse Range, Canterbury); seeds of *Sophora* spp. and *Clanthus puniceus* were purchased from New Zealand Tree Seeds, Rangiora, NZ and

seeds of *Astragalus sinicus* obtained from the Margot Forde Germplasm Centre, Palmerston North, NZ. All plant procedures carried out until processing of nodules, took place under sterile conditions. All seeds were, in sequence, soaked in concentrated sulphuric acid for 30–90 minutes, rinsed with sterile water then soaked in hot (~60°C) sterile water which was left at room temperature overnight. Seeds were then transferred to water agar plates. After germination, seedlings were transferred to polyeth-



Figure 1. 16S rRNA gene phylogenetic tree of bacterial strains isolated from New Zealand native legumes and selected *Mesorhizobium* type strains. Ca=*Carmichaelia australis*; Cm=*Carmichaelia monroi*; Cn=*Carmichaelia nana*; Co=*Carmichaelia odorata*; Cp=*Carmichaelia petriei*; Clp=*Clanthus puniceus*; Mn=*Montigena novae-zelandiae*. ■ indicates strains focussed on in current study. Numbers on branches are bootstrap % from 500 replicates (shown only when ≥50%). The tree was rooted with the *Bradyrhizobium elkani* type strain. doi:10.1371/journal.pone.0047677.g001

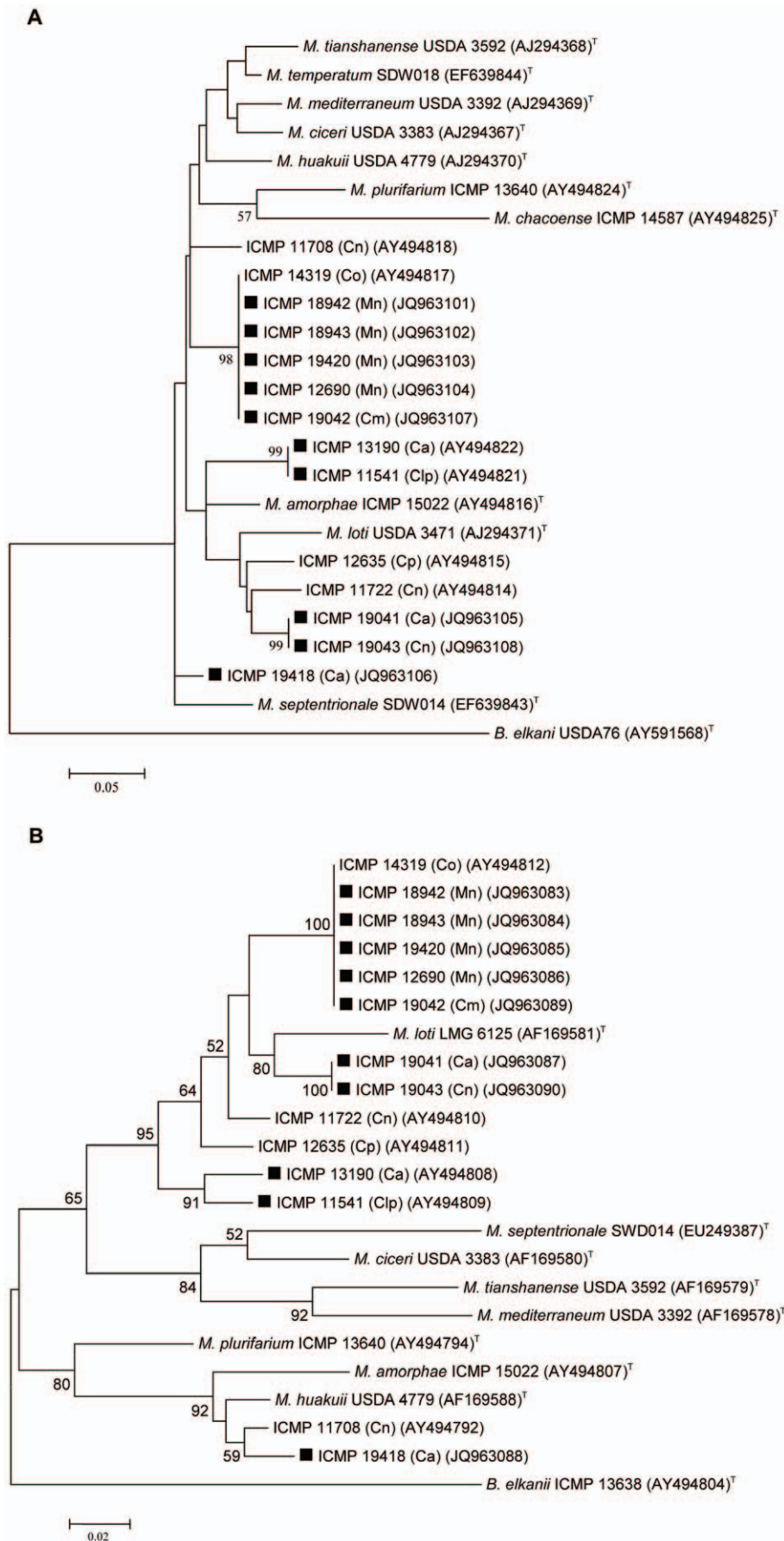


Figure 2. *recA* (a) and *glnII* (b) gene phylogenetic trees of bacterial strains isolated from New Zealand native legumes and selected *Mesorhizobium* type strains. Ca = *Carmichaelia australis*; Cm = *Carmichaelia monroi*; Cn = *Carmichaelia nana*; Co = *Carmichaelia odorata*; Cp = *Carmichaelia petriei*; Clp = *Clianthus puniceus*; Mn = *Montigena novae-zelandiae*. ■ indicates strains focussed on in this study. Numbers on branches are bootstrap % from 500 replicates (shown only when $\geq 50\%$). The trees were rooted with the *Bradyrhizobium elkani* type strain.
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ylene terephthalate jars (two seedlings per jar) containing vermiculite and supplied a complete nutrient medium (pH 6.0) containing NH_4NO_3 (0.1 mM), CaCl_2 (1.0 mM), KCl (1.0 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0 mM), NaH_2PO_4 (1.0 mM), Na_2HPO_4 (0.1 mM), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (5.0 μM), H_3BO_3 (5.0 μM), $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ (1.0 μM), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.5 μM), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1 μM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 μM) and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.02 μM). Plants were grown in a controlled environment cabinet and exposed to a 16 h photoperiod (400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at a constant 22°C.

At 5–15 days after sowing, seedlings were inoculated with the appropriate rhizobial strain grown to log phase: uninoculated plants supplied YMB only were used as controls. There were at least 3 replicate jars per treatment. Plants were inspected at two weekly intervals for nodulation and at 30–50 days after inoculation were tested for nitrogenase activity using the acetylene reduction assay (ARA) [31]. After the ARA, rhizobial strains were isolated from three to six nodules per treatment and their 16S rRNA gene sequenced.

Results

All ten bacterial strains isolated from *Carmichaelia*, *Clianthus* or *Montigena* studied here clustered closely with the *M. huakuii* type strain and five other strains previously isolated from *Carmichaelia* or *Clianthus* spp. (ICMP 11708, ICMP 14319, ICMP 12680, ICMP 11722 and ICMP 12635) [16,32], on the basis of their 16S rRNA sequences (Fig. 1). Eight strains, ICMP 18942, ICMP 18943, ICMP 19042, ICMP 19420, ICMP 12690, ICMP 19041 and ICMP 19418 this study and ICMP 11708 were identical and showed 99.5% similarity (1200 bp) to the *M. huakuii* type strain.

The ten strains separated into four groups on the basis of their *recA* sequences with all four groups clearly separated from all *Mesorhizobium* type strains (Fig. 2a). The four groupings on the basis of the *recA* sequences held in relation to *glnII* sequences (Fig. 2b). For *glnII* sequences, three groups (nine strains) were most closely related to, but clearly separated from, the *Mesorhizobium loti* type strain while strain ICMP 19418 (Ca) was most closely related to *M. huakuii*. Three other strains previously isolated from *Carmichaelia*

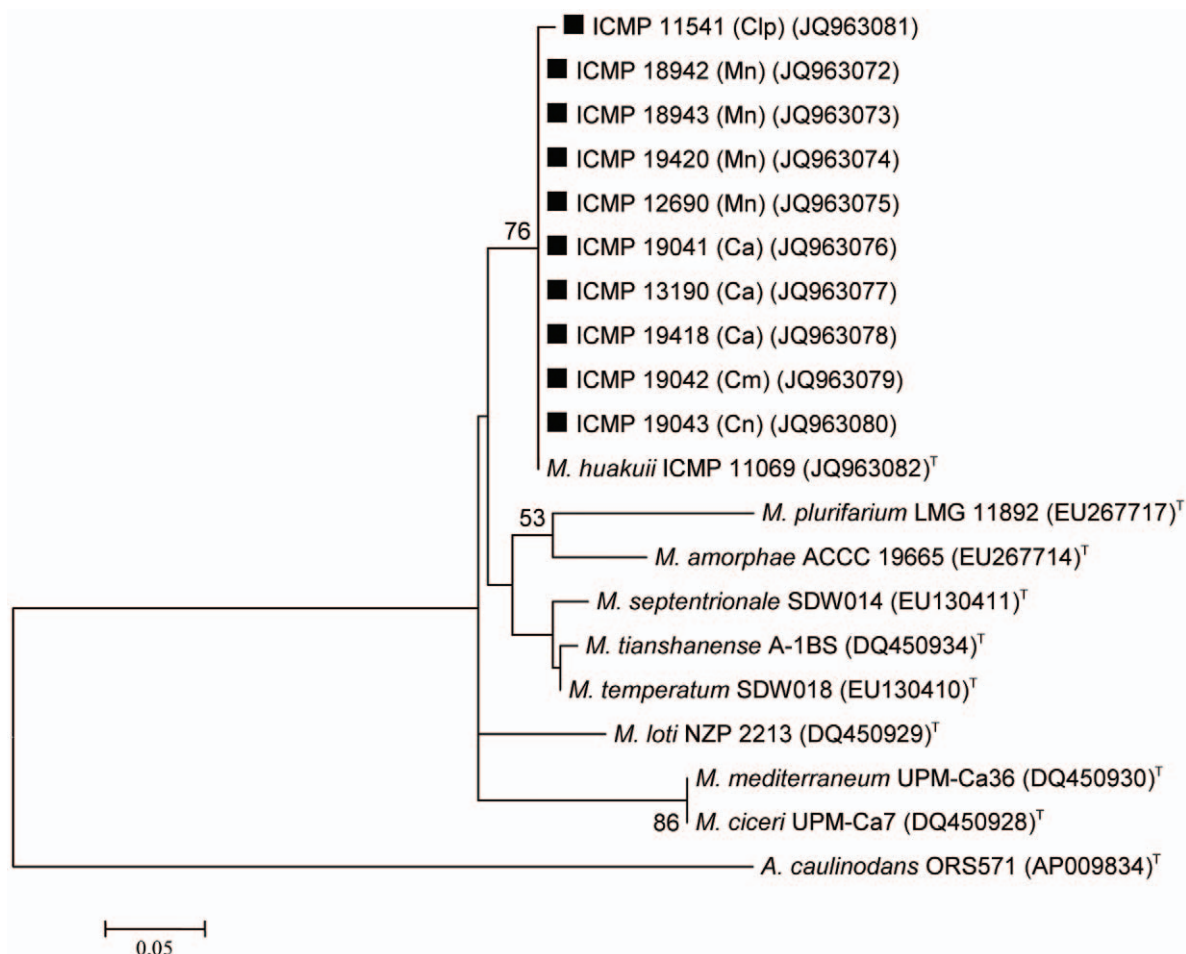


Figure 3. *nifH* gene phylogenetic tree of bacterial strains isolated from New Zealand native legumes and selected *Mesorhizobium* type strains. Ca = *Carmichaelia australis*; Cm = *Carmichaelia monroi*; Cn = *Carmichaelia nana*; Clp = *Clianthus puniceus*; Mn = *Montigena novae-zelandiae*. ■ indicates strains focussed on in this study. Numbers on branches are boot strap % from 500 replicates (shown only when $\geq 50\%$).
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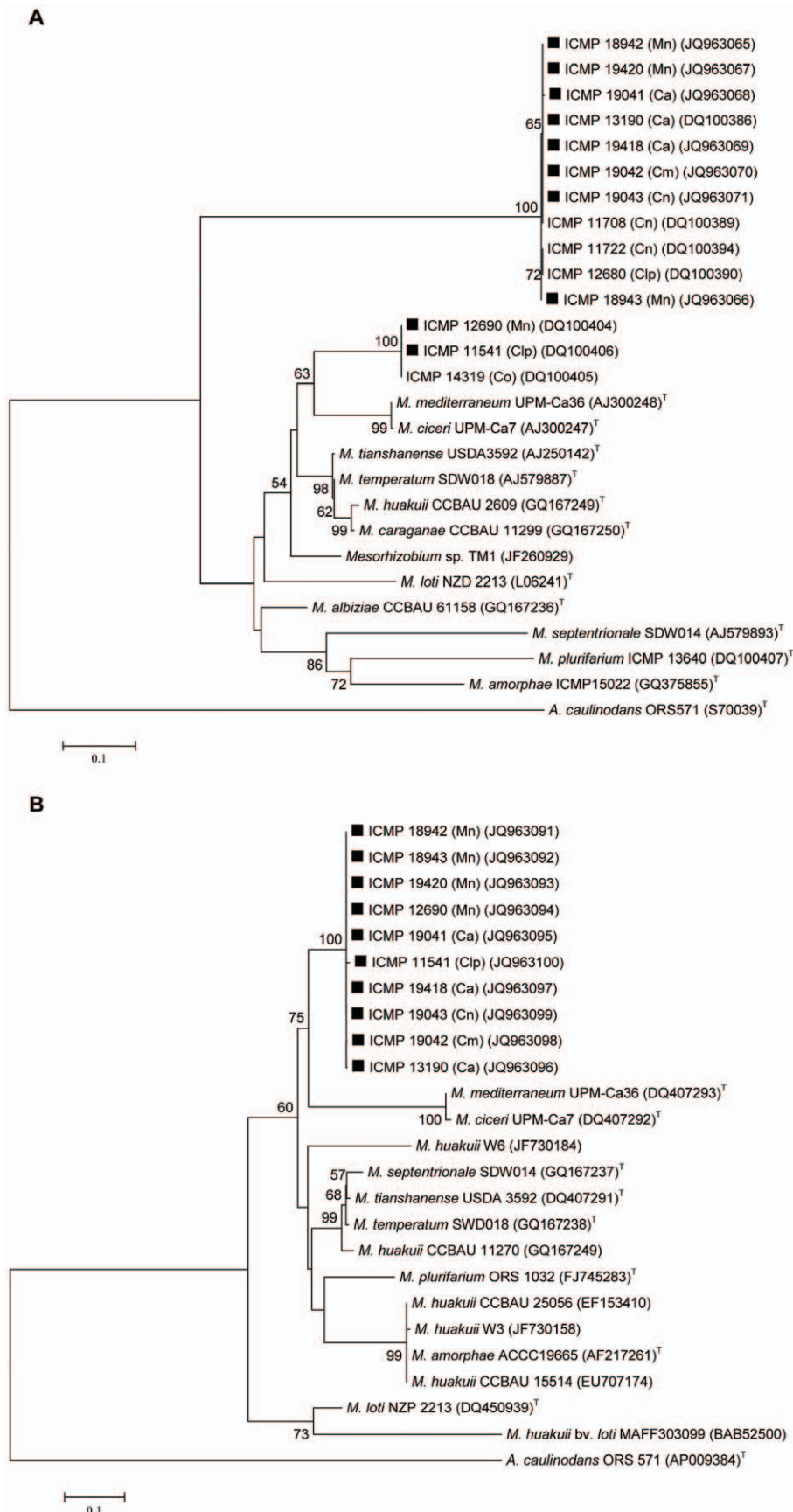


Figure 4. *nodA* (a) and *nodC* (b) gene phylogenetic trees of bacterial strains isolated from New Zealand native legumes, selected *Mesorhizobium* type strains and strains characterised as *Mesorhizobium huakuii* on the basis of their 16S rRNA sequences. Ca = *Carmichaelia australis*; Cm = *Carmichaelia monroi*; Cn = *Carmichaelia nana*; Co = *Carmichaelia odorata*; Clp = *Clanthus puniceus*; Mn = *Montigena novae-zelandiae*. ■ indicates strains focussed on in this study. Numbers on branches are bootstrap % from 500 replicates (shown only when $\geq 50\%$). doi:10.1371/journal.pone.0047677.g004

spp. (ICMP 14319, ICMP 11722 and ICMP 12635) [16] also aligned closest to *M. loti*.

There was little variation in the *nifH* gene sequences across the ten strains studied (Fig. 3). With the exception of ICMP 11541 (Clp), all strains were identical (290 bp) to the *M. huakuii* type strain (sequenced in the current study). The ten strains separated into two groups on the basis of their *nodA* sequences (Fig. 4a). The larger group (eight strains) clustered with three other strains (ICMP 11708, ICMP 11722 and ICMP 12680) previously isolated from *Carmichaelia* spp. or *Clanthus puniceus* [32], while the smaller group (two strains) grouped with strain ICMP 14319 isolated from *Carmichaelia odorata* [32]. The *nodA* sequences for the two groups showed only 66.29% similarity (530 bp) to each other but both separated clearly from all *Mesorhizobium* type strains and strain TMI isolated from *Thermopsis lupinoides* and characterised as *M. huakuii* on the basis of its 16S rRNA sequence [30].

All ten strains grouped closely together in relation to their *nodC* sequences and as for *nodA* sequences separated from all others on the Genbank database including those of strains characterised as *M. huakuii* on the basis of their 16S rRNA sequences (Fig. 4b) [33,34].

All seven *Carmichaelinae* strains tested and the *M. huakuii* type strain produced functional nodules on *Carmichaelia australis*, *Carmichaelia kirkii*, *Carmichaelia petriei*, *Clanthus puniceus* and *Astragalus sinicus* but did not nodulate either of the two *Sophora* species (Table 2). In all cases, the 16S rRNA sequence for the strain recovered from nodules after the acetylene reduction assay was identical to that of the strain used as inoculant.

Discussion

Genotypic data on the rhizobia which induce nodules on NZ native legumes are limited but it seems likely that rhizobial symbionts co-evolved with NZ native legumes in isolation from the regions of major legume evolution and as a result, may have novel characteristics. Here we focus on ten bacterial strains isolated from NZ native legumes in the current and previous studies which

closely aligned with *M. huakuii* on the basis of their 16S rRNA sequences. It is shown that the ten strains also grouped closely to the *M. huakuii* type strain in relation to their *nifH* sequences. However, with the exception of *glnII* for strain ICMP 19418 (Ca), all sequences for *recA*, *glnII*, *nodA* and *nodC* were substantially different for the ten strains and the *M. huakuii* type strain. The ten strains separated into the same four groups on the basis of their *recA* and *glnII* sequences. For *recA*, the four groups clearly separated from all *Mesorhizobium* type strains. For *glnII*, three groups (nine strains) aligned closest to but clearly separated from the *M. loti* type strain. It is possible that *Mesorhizobium* evolved in NZ with variable *recA* and *glnII* genes. However, many legume species have been introduced and have naturalised in NZ since colonisation by Europeans in the 19th Century. Also, in the case of crop legumes, rhizobial inoculant, was often applied, and in some cases still is, and it has been shown that chromosomal symbiosis genes can transfer ('lateral' transfer) from a *Mesorhizobium loti* inoculant strain to indigenous *M. huakuii* strains in NZ soils [35]. Thus, the possibility of genetic exchange of *recA* and *glnII* genes between recently introduced rhizobia and the indigenous *Mesorhizobium* should be considered. Certainly, there is strong evidence that in the past, horizontal transfer of the *glnII* gene has occurred between major rhizobial groups [24,36], but it would require substantial further work to determine if this has happened between rhizobia in NZ over the past 150 years.

All the strains grouped closely together in relation to their *nodC* sequences and these sequences separated from all others in Genbank. There was no *nodC* sequence for the *M. huakuii* type strain in Genbank and we were unable to sequence it. However, the *nodC* sequences for the strains separated from those of other strains characterised as *M. huakuii* on the basis of their 16S rRNA sequences. The ten strains separated into two groups in relation to their *nodA* sequences, eight of the strains studied grouped with three other strains previously isolated from *Carmichaelia* spp. or *Clanthus puniceus* while two strains grouped with a strain isolated from *Carmichaelia odorata* [32]. The *nodA* sequences for both groups separated clearly from those of all the *Mesorhizobium* type strains

Table 2. Host specificity of rhizobial strains used in this study.

Strain	Species tested						
	<i>Carmichaelia</i>			<i>Sophora</i>		<i>Clanthus</i>	<i>Astragalus</i>
	<i>australis</i>	<i>kirkii</i>	<i>petriei</i>	<i>microphylla</i>	<i>tetraptera</i>	<i>puniceus</i>	<i>sinicus</i>
ICMP 18942 (Mn*)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
ICMP 12690 (Mn)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
ICMP 19041 (Ca)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
ICMP 19042 (Cm)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
ICMP 19418 (Ca)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
ICMP 13190 (Ca)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
ICMP 11541 (Clp)	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+
<i>M. huakuii</i>	Nod+Fix+	Nod+Fix+	Nod+Fix+	Nod—	Nod—	Nod+Fix+	Nod+Fix+

*Original host: Mn = *Montigena novae-zelandiae*; Ca = *Carmichaelia australis*; Cm = *Carmichaelia monroi*; Clp = *Clanthus puniceus*.

Nod— = no plants nodulated; Nod+ = all plants nodulated; Fix+ = nitrogen fixing nodules.

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and the 'novel' *nodA* sequence reported for the *M. huakuii* strain isolated from *Thermopsis lupinoides* in Kamchatka, Russia [30]. Thus, although the *nifH* gene of the ten strains aligns closely with that of *M. huakuii*, their *nodC* and *nodA* genes are novel, and it seems likely that they evolved in rhizobia indigenous to NZ.

All seven strains selected (isolated from three separate genera, over three separate studies and including isolates from the North and South Islands of NZ) produced functional nodules on the three *Carmichaelia* spp. and *Clianthus puniceus* tested indicating that this group of bacteria are common rhizobial symbionts of *Carmichaelinae* species in NZ. None of the strains produced functional nodules on the *Sophora* spp. tested indicating that, within NZ native legumes, they are specific to *Carmichaelinae* species. Host range in rhizobia is at least in part determined by the structure of the lipo-chitin oligosaccharides 'nod factors' synthesised by the products of nodulation genes such as *nodA* and *nodC* [31,37]. Thus, it is to some extent unexpected that, although the *nodA* and *nodC* genes of the strains are novel, all seven strains tested, produced functional nodules on *Astragalus sinicus*, and that the *M. huakuii* type strain isolated from *A. sinicus* produced functional nodules on the *Carmichaelia* spp. and *Clianthus puniceus*. As the strains form nodules on *Astragalus sinicus*, they do not meet the criteria required to be considered as a different *M. huakuii* symbiovar [38].

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Author Contributions

Conceived and designed the experiments: MA HWT PBH HJR. Performed the experiments: HWT MA PBH HJR. Analyzed the data: MA HWT PBH JPWY HJR EKJ JIS BSW NC. Contributed reagents/materials/analysis tools: NC BSW. Wrote the paper: MA HWT PBH HJR.

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