
http://researchrepository.murdoch.edu.au/25307/

Copyright: © 2014 Elsevier GmbH.

It is posted here for your personal use. No further distribution is permitted.
Accepted Manuscript

Title: Diverse novel mesorhizobia nodulate New Zealand native *Sophora* species

Author: Heng Wee Tan Peter B. Heenan Sofie E. De Meyer Anne Willems Mitchell Andrews

PII: S0723-2020(14)00175-1
DOI: http://dx.doi.org/doi:10.1016/j.syapm.2014.11.003
Reference: SYAPM 25664

To appear in:

Received date: 25-10-2014
Revised date: 12-11-2014
Accepted date: 14-11-2014

Please cite this article as: H.W. Tan, P.B. Heenan, S.E. De Meyer, A. Willems, M. Andrews, Diverse novel mesorhizobia nodulate New Zealand native *Sophora* species, *Systematic and Applied Microbiology* (2014), http://dx.doi.org/10.1016/j.syapm.2014.11.003

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Diverse novel mesorhizobia nodulate New Zealand native *Sophora* species

Heng Wee Tan\textsuperscript{a}, Peter B. Heenan\textsuperscript{b}, Sofie E. De Meyer\textsuperscript{c}, Anne Willems\textsuperscript{d}, Mitchell Andrews\textsuperscript{a*}

\textsuperscript{a}Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, NZ
\textsuperscript{b}Allan Herbarium, Landcare Research, Lincoln 7640, NZ
\textsuperscript{c}Centre for Rhizobium Studies, Murdoch University, Murdoch, Western Australia 6150, Australia
\textsuperscript{d}Department of Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

**A B S T R A C T**

Forty eight rhizobial isolates from New Zealand (NZ) native *Sophora* spp. growing in natural ecosystems were characterised. Thirty eight isolates across five groups showed greatest similarity to *Mesorhizobium ciceri* LMG 14989\textsuperscript{T} with respect to their 16S rRNA and concatenated recA, glnl and rpoB sequences. Seven isolates had a 16S rRNA sequence identical to *M. amorphae* ATCC 19665\textsuperscript{T} but showed greatest similarity to *M. septentrionale* LMG 23930\textsuperscript{T} on their concatenated recA, glnl and rpoB sequences. All isolates grouped closely together for their nifH, nodA and nodC sequences, clearly separate from all other rhizobia in the Genbank database. None of the type strains closest to the *Sophora* isolates based on 16S rRNA sequence similarity nodulated *Sophora microphylla* but they all nodulated their original host. Twenty one *Sophora* isolates selected from the different 16S rRNA groupings produced N\textsubscript{2}-fixing nodules on three *Sophora* spp. but none nodulated any host of the type strains for the related species. DNA hybridisations indicated that these isolates belong to novel *Mesorhizobium* spp. that nodulate NZ native *Sophora* species.

**Keywords:** *Mesorhizobium*<br>*Sophora*<br>Phylogeny<br>Symbiosis genes

**Introduction**

Most legumes (plant family Fabaceae) have the capacity to fix atmospheric nitrogen (N\textsubscript{2}) via symbiotic bacteria (generally termed ‘rhizobia’) in root nodules which gives them an advantage under low soil nitrogen (N) conditions if other factors are favourable for growth [1,2,21]. There are four genera of native legume of the sub-family Papilionoideae on the main New Zealand (NZ) islands. These genera are the closely related *Carmichaelia* (23 endemic species), *Clianthus* (2 endemic species) and *Montigena* (1 endemic species) in the ‘Carmichaelinae’ clade, tribe Galegeae, and *Sophora* (8 endemic species) in the tribe Sophoreae [11,12,13,27]. All four genera are capable of forming nodules [21], but genotypic data on the rhizobia which induce nodules on the *Sophora* spp. are limited.

---

\* Corresponding author. Tel.: +64 3 4230692<br>E-mail address: Mitchell.andrews@lincoln.ac.nz
Previously, sequences were obtained for the small subunit ribosomal RNA (16S rRNA) gene of five isolates of *Sophora* spp. growing in natural ecosystems [34]. Four isolates were most closely grouped to the *Mesorhizobium ciceri* and *M. loti* type strains while the fifth isolate, aligned closely with *Rhizobium leguminosarum* strains. This study did not assess the ability of the isolated strains to nodulate NZ native legumes or sequence any of their N2-fixation (*nif*) or nodulation (*nod*) genes but subsequent work showed that although the *R. leguminosarum* strain (ICMP 14642) produced nodules on *Sophora microphylla*, these nodules did not fix N2 [33, http://scd.landcareresearch.co.nz].

Tan et al. [25] examined ten bacterial strains isolated from NZ *Carmichaelinae* growing in natural ecosystems which grouped close to the *M. huakuii* type strain in relation to their 16S rRNA and nitrogenase iron protein gene (*nifH*) sequences. These strains showed novel DNA recombinase A gene (*recA*), glutamine synthetase II gene (*glnll*), N-acyttransferase nodulation protein A gene (*nodA*) and N-acetylglicosaminytransferase nodulation protein C gene (*nodC*) sequences. Seven strains selected produced functional nodules on *Carmichaelia* spp. and *Cianthus puniceus* but did not nodulate two *Sophora* species indicating that within NZ native legumes, at least some bacterial strains are specific to *Carmichaelinae* species. Tan et al. [24] tested the ability of eleven isolates, two from *Montigena* and three each from *Carmichaelia* spp., *Cianthus puniceus* and *Sophora* spp. to nodulate *Montigena*. Two of the *Sophora* strains were taken from a previous study [34] and showed 16S rRNA similar to *M. loti*/*M. ciceri* but the third had a 16S rRNA sequence similar to *M. huakuii*. All three isolates from *Sophora* spp. were shown to nodulate their original host. Only isolates from *Carmichaelia* spp. and *Cianthus puniceus* that produced functional nodules on their original host and strains from *Montigena* produced functional nodules on *Montigena*. Strains that nodulated *Montigena*, *Carmichaelia* spp. and *Cianthus puniceus* had variable 16S rRNA, *recA* and *glnll* gene sequences, but specific *nifH*, *nodA* and *nodC* gene sequences different from those of the *Sophora* isolates. These results indicate that *Montigena*, *Carmichaelia* spp. and *Cianthus* spp. share at least some rhizobia with each other but not with *Sophora* spp. and that the ability of different rhizobial strains to produce functional nodules on the different NZ native legume genera is associated with specific symbiosis genes. Here we focus on bacterial isolates from *Sophora* spp. growing in natural ecosystems and characterise them with respect to their 16S rRNA, *recA*, *glnll*, *rpoB*, *nifH*, *nodA* and *nodC* gene sequences, ability to nodulate a range of legume species and DNA hybridisation tests with their most closely related rhizobial type strains on the basis of gene sequence similarity.

### Materials and methods

#### Bacterial isolates

Forty five bacterial isolates from *Sophora* spp. sampled in April 2011 or April 2012 under natural conditions in the current study plus the three *Sophora* isolates previously shown to nodulate their host [24] were studied. Here, bacteria were isolated from plants sampled at six sites in the South Island: alluvial limestone river terrace, Waima/Ure River, Marlborough (41° 52´S 174° 0´E, 147m; Field site 1); alluvial outwash river fan, Pororari River, Westland (42° 6S´ 171° 20´E, 1m; Field site 2); margin of estuary, Saltwater Creek, Greymouth, Westland (42° 30´S 171° 9´E, 2m; Field site 3); margin of Greywacke rock outcrop, Kowai River, Springfield, Canterbury (43° 19´S 171° 46´E, 612m; Field site...
4); alluvial Greywacke river terrace, upper Rakaia River, Canterbury (43° 26´S 171° 34´E, 357m; Field Site 5) and among Haast Schist rock outcrop, Waitaki River, Otago (44° 53´S 170° 48´E, 126m; Field site 6). Field sites were selected to represent low rainfall in the eastern South Island (< 1000 mm per year; sites 1, 4-6) and high rainfall in the western South Island (> 2500 mm per year, sites 2, 3) [16]. All isolates are deposited in the International Collection of Microorganisms from Plants (ICMP), Landcare Research, Auckland, NZ. The ICMP number, *Sophora* species host and field site sampled are given for the isolates on the phylogenetic trees. The type strains of *M. amorphae* (ICMP 15022 = ACCC 19665T), *M. ciceri* (ICMP 13641 = LMG 14989 T), *M. huakuii* (ICMP 11069 = IAM 14158T) and *M. loti* (ICMP 4682 = LMG 6125T) were obtained from the ICMP collection directly.

For bacteria isolated in this study, root nodules were surface sterilised, crushed in sterile water and the bacterial suspension was streaked onto yeast mannitol agar (YMA) [26] and incubated at 25°C in the dark for 5 days as described previously [25]. A purified culture was obtained by sub-culture from each plate. Each culture was inoculated into a suspension of yeast mannitol broth (YMB) [26] and used for preparation of subcultures for DNA extraction or inoculum.

**DNA sequencing**

DNA was extracted from the bacterial cultures grown in YMB using the standard Qiagen-Gentra PUREGENE DNA Purification Kit for gram-negative bacteria. The 16S rRNA, *recA*, *glnl*, *nifH*, *nodA* and *nodC* genes were amplified with appropriate primer sets and PCR conditions as described previously [25] except that for most strains, the *nodC* primers NodCfor540 (TGA-TYG-AYA-TGG-ART-AYT-GGC-T) and NodCrev1160 (CGY-GAC-ARC-CAR-TCG-CTR-TTG) [20] were used. The *rpoB*83F (CCT- SAT-CGA-GGT -TCA-CAG-AAG-GC) and *rpoB*1061R (AGC-GTG -TTG-CGG-ATA-TAG-GCG) primers [17] were used for *rpoB* amplification. The PCR products were separated by electrophoresis in 1% (w/v) agarose gels, stained with ethidium bromide (0.5 μg mL⁻¹) and viewed under UV light. PCR products of the expected size were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University. DNA sequence data were viewed via Sequence Scanner v 1.0 software (©Applied Biosystems) and edited and assembled using DNAMAN Version 6 (©Lynnon Biosoft Corporation).

**Phylogenetic analyses**

DNA sequences of individual genes and concatenated *recA*, *glnl* and *rpoB* for the *Sophora* isolates were aligned and Maximum Likelihood (ML) trees constructed using the partial deletion method with an 80% cut off in MEGA5 software [23]. Bootstrap support for each node was evaluated with 500 replicates. The most closely related *Mesorhizobium* type strains on the Genbank sequence database [www.ncbi.nlm.nih.gov/genbank] were used for all trees. In addition, sequences of closely related non-type strains were included in the *nifH* and *nodC* trees and those of strains shown to produce N₂ fixing nodules on NZ *Carmichaelinae* species in the *nodA* and *nodC* trees. Only bootstrap values ≥50% are shown for each tree. 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. GenBank accession numbers for all genes are shown in the figures.
Nodulation and N\textsubscript{2} fixation studies

All bacterial strains isolated in the current study were inoculated onto their original host legume species. In addition, twenty one of the isolates of variable 16S rRNA, recA, glnl and rpoB gene sequence groups, but which all nodulated their host *Sophora* sp. were inoculated onto *Sophora microphylla*, *Sophora prostrata*, *Sophora longicarinata*, *Carmichaelia australis* and *Cianthus puniceus*. *Mesorhizobium amorphae* ACCC 19665\textsuperscript{T}, *M. ciceri* LMG 14989\textsuperscript{T}, *M. huakuii* IAM 14158\textsuperscript{T} and *M. loti* LMG 6125\textsuperscript{T} were inoculated onto the three *Sophora* species and the *Sophora* isolates were also inoculated onto *Amorpha fruticosa*, *Cicer arietinum*, *Astragalus sinicus* and *Lotus corniculatus* the legume species from which respectively, the *M. amorphae* [29], *M. ciceri* [18], *M. huakuii* [5] and *M. loti* [14] type strains were originally isolated. The type strains were inoculated onto their original host as a positive control. Seeds were obtained from different sources: *Carmichaelia australis* from Proseed, North Canterbury, NZ; *Cianthus puniceus* and all *Sophora* spp. from New Zealand Tree Seeds, Rangiora, NZ; *Amorpha fruticosa* from the Agroforestry Research Trust, Devon, UK; *Astragalus sinicus* and *Lotus corniculatus* from the Margot Forde Germplasm Centre, Palmerston North, NZ and *Cicer arietinum* from the Binn Inn, Christchurch, NZ.

All plant procedures were carried out under gnotobiotic conditions. All seeds, except those of *Cicer arietinum* were, in sequence, soaked in concentrated sulphuric acid for 30-60 minutes, rinsed with sterile water, soaked in hot (\(~ 60\textdegree C\)) sterile water which was left at room temperature overnight then transferred to 1.5% water agar plates and kept in the dark to germinate. After germination, each seedling was transferred to a polyethylene terephthalate jar containing vermiculite and supplied with a complete nutrient solution [25]. Plants were grown in a chamber with a 16 hour photoperiod (400 \(\mu\)mol photons m\textsuperscript{-2} s\textsuperscript{-1}) at a constant 22\textdegree C. Seeds of *Cicer arietinum* were soaked in 20% commercial bleach (0.5 g L\textsuperscript{-1} sodium hypochlorite) for 20 min, rinsed with 96% ethanol, air dried then soaked in hot sterile water and left to cool overnight. *Cicer arietinum* was germinated and grown in 0.75 L pots (four seedlings per pot) containing autoclaved N-free potting mix under natural daylight in a glasshouse. The potting mix base was 80% composted bark and 20% pumice (1-4 mm) to which was added 1 g L\textsuperscript{-1} agricultural lime (primarily calcium carbonate), 0.3 g L\textsuperscript{-1} superphosphate (9P-11S-20Ca; Ravensdown, NZ), and 0.3 g L\textsuperscript{-1} Osmocote (6 months, ON-OP-37K), 0.3 g L\textsuperscript{-1} Micromax trace elements and 1 g L\textsuperscript{-1} Hydraflo, all three obtained from Everris International, Geldermalsen, The Netherlands. The pH of the medium was 5.8.

At 5-10 days after sowing, seedlings were inoculated with 10 ml of the appropriate bacterial strain grown to log phase. Uninoculated plants supplied with YMB only were used as controls. There were 3 replicates per treatment. All plants were supplied with sterile water as required. Plants were inspected at three weekly intervals for nodulation and at 70-90 days after inoculation were tested for nitrogenase activity using the acetylene reduction assay (ARA) [6]. All values taken as negative were similar to controls and in the range 2.1 to 4.5 \(\mu\)L ethylene L\textsuperscript{-1}. All values taken as positive were one to two orders of magnitude greater than this (ranging from 198.9 to 2107.3 \(\mu\)L ethylene L\textsuperscript{-1}). After the ARA, rhizobial strains were isolated from three to six nodules per treatment and their 16S rRNA and
nodC genes sequenced. In all cases, the 16S rRNA and nodC sequences for the strain recovered from nodules from the inoculation assays were identical to those of the strain used as inoculant.

DNA-DNA hybridisation

For DNA-DNA hybridisation, high-molecular weight DNA was prepared [19]. DNA-DNA hybridisations were performed using a microplate method and biotinylated probe DNA [7]. The hybridisation temperature was 48 °C ± 1 °C. Reciprocal reactions (A × B and B × A) were performed in quadruplicate for each DNA pair and their variation was within the limits of this method [10].

Results and discussion

In the current study, forty eight bacterial isolates from Sophora spp. were shown to produce N2-fixing nodules on their host species. These isolates separated into three major groupings on the basis of their 16S rRNA sequences (Fig. 1). Group one, comprising thirty eight isolates from four different Sophora species across eight different field sites separated into five sub-groups with high similarity (99.60-100% similarity, 1260 bp) to M. ciceri LMG 14989T isolated from Cicer arietinum in Spain [18]. The second group, comprising seven isolates of Sophora longicarinata from Field site 1, showed 16S rRNA sequences (1260 bp) identical (100% similarity) to that of the M. amorphae type strain which was isolated from Amorpha fruticosa growing in Beijing, China, although there is evidence that its origin was the native range of Amorpha fruticosa which is South Eastern and Mid-Western USA [28,29]. The third group contains three isolates (ICMP 19550, ICMP 19559 and ICMP 19567) aligning closely, based on 16S rRNA gene analysis, with M. huakuii IAM 14158T which was isolated from Astragalus sinicus nodules sampled in Nanjing province, China [5]. These data indicate that Sophora spp. and Carmichaelinae species in NZ [24,25] can be nodulated by Mesorhizobium spp. with 16S rRNA gene sequences similar to M. ciceri, M. huakuii and M. amorphae.

The forty eight mesorhizobial isolates from Sophora spp. separated into eight groups and three individual isolates (ICMP 11719, ICMP 19513 and ICMP 19550) separate from all Mesorhizobium type strains. Generally, isolates from the same field site grouped together. This apparent link between recA, glnl and rpoB sequences and field site may be at least in part, due to adaptation of the bacteria to local conditions outside the host plant and this warrants further study. Seven of the groups and the three individual isolates (40 isolates) clustered around M. ciceri LMG 14989T (84.5-86.5 % similarity, 1800 bp), while one group of eight isolates, all from Sophora longicarinata sampled at field site 1, was closest to (87.9% similarity, 1800 bp) M. septentrionale LMG 23930T isolated from Astragalus adsurgens growing in Northern China [8]. This second group contained all seven isolates with a 16S rRNA sequence identical to M. amorphae ACCC 19665T and ICMP 19567 which had a 16S rRNA sequence closest to M. huakuii IAM 14158.

The nifH gene sequences for all forty eight isolates clustered together (97.4-100% similarity, 280 bp) closest to but separate from (92.5-95.6% similarity, 280 bp) M. alhagi CCNWXJ12-2T isolated from Alhagi sparsifolia in Xinjiang province, China [3]; Mesorhizobium strain CCNWSX672 isolated from Coronilla varia in Shaanxi province, China [35]; and M. camelthorni CCNWX340-4T isolated from
Alhagi sparsifolia in Xinjiang Province, China [4] (Fig. 3). The nifH sequences of the forty eight Sophora isolates differed (92.5-94.7% similarity, 280 bp) from the nifH sequences of the seven strains previously shown to induce N₂ fixing nodules on Carmichaelinae but not Sophora species which were identical (six strains) or very similar to that of the M. huakuii type strain [24,25].

The nodA sequences for the Sophora isolates showed 97.4-100% similarity to each other (420 bp) and clearly separated from those of all Mesorhizobium type strains and rhizobial symbionts of NZ Carmichaelinae species (Fig. 4A). The strains aligned closest to (86.5-88.4% similarity, 420 bp) the M. albiziae type strain isolated from Albizia kalkora in Suchian province, Southern China [30,31]. The nodC sequences for the Sophora isolates showed 98.9-100% similarity to each other (650 bp) and as for nodA sequences were clearly separated from all Mesorhizobium type strains and rhizobial symbionts of NZ Carmichaelinae species (Fig. 4B). Here, the isolates aligned closest to M. albizeae CCBAU 61158T (91.5-91.7% similarity, 650 bp) and Mesorhizobium strain CBAU03074 (93.5-94.2% similarity, 650 bp) isolated from Astragalus scaberrimus in temperate China which had a 16S rRNA sequence identical to M. septentrionale [37]. Overall, the data obtained in the current and previous studies provide strong evidence that mesorhizobia are the major, if not exclusive, rhizobial partners of Sophora spp. in NZ. These mesorhizobia had variable 16S rRNA, recA, glnI and rpoB but specific nifH, nodA and nodC gene sequences. A similar generalisation was made for rhizobia that nodulate NZ Carmichaelinae species [25] but the symbiosis genes of the two groups are very different from each other. Considering work on Sophora outside NZ, 16S rRNA and recA gene sequences of 75 rhizobial isolates indicated that Mesorhizobium sp., Agrobacterium sp., Ensifer sp., Phyllobacterium sp. and Rhizobium sp. could effectively nodulate Sophora alopecuroides grown in different regions of the Loess Plateau in China [36]. These isolates had diverse nifH and nodA genes similar to those of rhizobial isolates from a range of legume genera in the same region indicating that these legumes may be able to share these rhizobia. However, as for rhizobia from NZ Sophora, there was a correlation between genotype and geographical origin for rhizobia isolated from Sophora alopecuroides.

Previously, rhizobia shown to produce N₂ fixing nodules on Carmichaelinae species did not nodulate NZ Sophora spp. [24,25]. Here, twenty one Sophora isolates from the different 16S rRNA/housekeeping gene groupings were inoculated on Sophora microphylla, Sophora prostrata, Sophora longicarinata, Clianthus puniceus and Carmichaelia australis (Table 1). All Sophora rhizobial isolates tested, regardless of their 16S rRNA or concatenated recA, glnI and rpoB gene grouping, produced N₂ fixing nodules on the three Sophora species but none induced N₂ fixing nodules on Carmichaelia australis. However, eighteen of the twenty one isolates produced N₂ fixing nodules on Clianthus puniceus. Clianthus puniceus, therefore, can share some rhizobia with Sophora spp. and others with Carmichaelia spp. (Table 1) and Montigena [24]. Host range in rhizobia is at least in part determined by the structure of the lipo-chitin oligosaccharide ‘Nod factors’ synthesised by the products of the nodulation genes such as nodA and nodC [6,15]. Therefore, the substantial differences in the nodA and nodC gene sequences between isolates that produce N₂ fixing nodules on NZ Sophora spp. or Carmichaelia spp. and Montigena are likely to be important in determining the specificity of the different groups of strains. It is thus unexpected that rhizobial symbionts of Sophora
spp., and *Carmichaelia* spp. and *Montigena* can induce functional nodules on *Clianthus puniceus*. This indicates that *Clianthus puniceus* is a more promiscuous rhizobial host than *Carmichaelia* spp., *Montigena* or *Sophora* spp. Previously, *M. huakuii* IAM 14158\(^T\) was shown to nodulate *Carmichaelia* spp. and *Clianthus puniceus* [25]. Here, none of the type strains closest to the *Sophora* isolates on 16S rRNA sequences (*M. ciceri*, *M. amorphae* or *M. huakuii* depending on strain) nor the *M. loti* type strain, isolated from nodules of *Lotus corniculatus* in Wanganui, NZ [14], produced nodules on *Sophora microphylla*, *Sophora prostrata* or *Sophora longicarinata* (Table 1) but they all induced N\(_2\) fixing nodules on their original host (data not shown). Also, none of the 21 *Sophora* isolates nodulated any of the type strain hosts (data not shown) indicating a specific symbiosis with *Sophora* spp.

It is generally accepted that strains within a species should exhibit more than 70% DNA hybridisation [9,22,32]. In the current study, DNA-DNA hybridisation values ranged from 30.1-52.1% between the five *Sophora* isolates selected from the different 16S rRNA/ housekeeping gene groups indicating that these groups are quite different from each other (Table 2). Additionally, these strains showed only 26.1-36.3% similarity to *M. ciceri* LMG 14989\(^T\). Also, isolate ICMP 19515 which was identical to *M. ciceri* LMG 14989\(^T\) on 16S rRNA sequence and closest to *M. ciceri* on concatenated housekeeping gene sequences showed only 28.9% similarity to *M. ciceri* LMG 14989\(^T\). Similarly, strain ICMP 19557 which had a 16S rRNA sequence identical to *M. amorphae* ACCC 19665\(^T\) and showed greatest similarity to *M. septentrionale* LMG 23930 on concatenated *recA*, *glnII* and *rpoB* gene sequences showed 28.8% similarity to *M. septentrionale* LMG 23930\(^T\) and 32.3% similarity to ICMP 19515. Thus, DNA-DNA hybridisations indicate that the isolates belong to novel *Mesorhizobium* spp.

In this study, DNA sequencing, DNA hybridisations and inoculation experiments have revealed that a significant diversity of novel *Mesorhizobium* spp. nodulate NZ native *Sophora* spp. The ‘drivers’ for this diversity are unknown and require further study. Abiotic characteristics of the sampling sites may be important drivers as the field sites represent a variety of South Island habitats, including parent rock type (e.g. schist, greywacke and limestone), substrate (alluvium and rock outcrop), and rainfall (> 2500 mm in western South Island, < 1000 mm in eastern South Island).

**Acknowledgements**

This work was supported by the Brian Mason Scientific and Technical Trust and a Lincoln University Doctoral Scholarship (HWT). We thank Liesbeth Lebbe for excellent technical assistance.

**References**


Legends to Figures

Fig. 1. 16S rRNA gene maximum likelihood (ML) tree (ca. 1260 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type strains. SI = *Sophora longicarinata*; Sm = *Sophora microphylla*; Sp = *Sophora prostrata*; St = *Sophora tetraptera*. The Kimura 2-parameter + Gamma distribution (K2 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 500 replicates (shown only when ≥ 50%). The tree was rooted with *Bradyrhizobium elkani* USDA 761. Scale bar = 2% sequence divergence (2 substitutions per 100 nucleotides). *Isolated in previous study [34]. FS = field site.

Fig. 2. Concatenated recA, *gln*II and *rpoB* gene maximum likelihood (ML) tree (ca. 1800 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type strains. SI = *Sophora longicarinata*; Sm = *Sophora microphylla*; Sp = *Sophora prostrata*; St = *Sophora tetraptera*. The Tamura-Nei with gamma distribution (TN93 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 500 replicates (shown only when ≥ 50%). The trees were rooted with *Bradyrhizobium elkani* USDA 761. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). *Isolated in previous study [34]. FS = field site.

Fig. 3. *nifH* gene maximum likelihood (ML) tree (ca. 280 bp) of bacterial strains isolated from New Zealand native *Sophora* spp. and selected *Mesorhizobium* type and non-type strains. SI = *Sophora longicarinata*; Sm = *Sophora microphylla*; Sp = *Sophora prostrata*; St = *Sophora tetraptera*. The Tamura 3-parameter with gamma distribution (T92 + G) model was used to construct the tree. Numbers on branches are bootstrap % from 500 replicates (shown only when ≥ 50%). The trees were rooted with *Azorhizobium caulindans* ORS5711. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). *Isolated in previous study [34]. FS = field site.

Fig. 4. *nodA* (A) and *nodC* (B) gene maximum likelihood (ML) trees (ca. 420 bp and 650 bp respectively) of bacterial strains isolated from New Zealand native *Sophora* spp., selected *Mesorhizobium* type and non-type strains and strains shown to produce functional nodules on NZ Carmichaeliaceae species (superscript C). SI = *Sophora longicarinata*; Sm = *Sophora microphylla*; Sp = *Sophora prostrata*; St = *Sophora tetraptera*. The Tamura 3-parameter with gamma distribution and invariant sites (T92 + G + I) model was used to construct the trees. Numbers on branches are bootstrap % from 500 replicates (shown only when ≥ 50%). The trees were rooted with *Azorhizobium caulindans* ORS5711. Scale bars = 1% sequence divergence (1 substitution per 100 nucleotides). *Isolated in previous study [34]. FS = field site.

Page 10 of 16
Table 1. Host specificity of rhizobial strains used in this study.

<table>
<thead>
<tr>
<th>16S rRNA grouping</th>
<th>Strain</th>
<th>Host</th>
<th>Sophora microphylla</th>
<th>Sophora prostrata</th>
<th>Sophora longicarinata</th>
<th>Clionthus puniceus</th>
<th>Carmichaelia australis</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. ciceri</td>
<td>ICMP 11719</td>
<td>S. tetraptera</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 14330</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19514</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19515</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19519</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19520</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19560</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19561</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19545</td>
<td>S. prostrata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19546</td>
<td>S. prostrata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19523</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19528</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. ciceri</td>
<td>ICMP 19535</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. amorphae</td>
<td>ICMP 19556</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. amorphae</td>
<td>ICMP 19558</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. amorphae</td>
<td>ICMP 19568</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. amorphae</td>
<td>ICMP 19569</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod-</td>
</tr>
<tr>
<td>M. huakuii</td>
<td>ICMP 19559</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. huakuii</td>
<td>ICMP 19567</td>
<td>S. longicarinata</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. huakuii</td>
<td>ICMP 19550</td>
<td>S. microphylla</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix+</td>
<td>Nod+ Fix-</td>
</tr>
<tr>
<td>M. ciceri&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ICMP 13641</td>
<td>Cicer arietinum</td>
<td>Nod-</td>
<td>Nod-</td>
<td>Nod-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M. amorphae&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ICMP 15022</td>
<td>Amorpha fruticosa</td>
<td>Nod-</td>
<td>Nod-</td>
<td>Nod-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M. huakuii&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ICMP 11069</td>
<td>Astragalus sinicus</td>
<td>Nod-</td>
<td>Nod-</td>
<td>Nod-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M. loti&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ICMP 4682</td>
<td>Lotus corniculatus</td>
<td>Nod-</td>
<td>Nod-</td>
<td>Nod-</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Nod+ = all plants nodulated; Nod- = no plants nodulated; Fix+ = N₂ fixing nodules; Fix- = nodules not fixing N₂.

N.D. Not determined
Table 2. % DNA-DNA hybridisation between pairs of *Sophora* isolates and *Sophora* isolates and their closest *Mesorhizobium* type strain.

<table>
<thead>
<tr>
<th>Strains</th>
<th>ICMP 19512</th>
<th>ICMP 19535</th>
<th>ICMP 19523</th>
<th>ICMP 19545</th>
<th>ICMP 19560</th>
<th>LMG 14989</th>
<th>ICMP 19515</th>
<th>ICMP 19557</th>
<th>LMG 23930</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICMP 19512</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICMP 19535</td>
<td>43.6</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICMP 19523</td>
<td>52.1</td>
<td>38.2</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICMP 19545</td>
<td>41.1</td>
<td>32.4</td>
<td>32.8</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICMP 19560</td>
<td>36.1</td>
<td>30.1</td>
<td>36.6</td>
<td>38.4</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. ciceri</em> LMG14989&lt;sup&gt;T&lt;/sup&gt;</td>
<td>29.2</td>
<td>31.1</td>
<td>27.0</td>
<td>26.1</td>
<td>36.3</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICMP 19515</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>28.9</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICMP 19557</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>29.2</td>
<td>31.8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>M. septentrionale</em> LMG 23930&lt;sup&gt;T&lt;/sup&gt;</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>32.3</td>
<td>28.8</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

N.D. Not determined
Fig. 1

16S rRNA

- ICMP 19526 (Sm) (KC237416) FS6
- ICMP 19540 (Sm) (KC237429) FS2
- ICMP 19539 (Sm) (KC237428) FS2
- ICMP 19538 (Sm) (KC237427) FS2
- ICMP 19537 (Sm) (KC237426) FS2
- ICMP 19536 (Sm) (KC237425) FS2
- ICMP 19535 (Sm) (KC237424) FS2
- ICMP 19532 (Sm) (KC237422) FS3
- ICMP 19531 (Sm) (KC237421) FS3
- ICMP 19530 (Sm) (KC237420) FS3
- ICMP 19529 (Sm) (KC237419) FS3
- ICMP 19528 (Sm) (KC237418) FS3
- ICMP 19527 (Sm) (KC237417) FS6
- ICMP 19525 (Sm) (KC237415) FS6
- ICMP 19524 (Sm) (KC237414) FS6
- ICMP 19523 (Sm) (KC237413) FS6
- ICMP 19521 (Sm) (KC237404) FS4
- ICMP 19520 (Sm) (KC237403) FS4
- ICMP 19519 (Sm) (KC237402) FS4
- ICMP 19518 (Sm) (KC237401) FS5
- ICMP 19517 (Sm) (KC237399) FS5
- ICMP 19516 (Sm) (KC237398) FS5
- ICMP 19515 (Sm) (KC237397) FS5
- ICMP 19514 (Sm) (KC237396) FS5
- ICMP 19560 (Sl) (KC237406) FS1
- ICMP 19561 (Sl) (KC237407) FS1
- ICMP 19562 (Sl) (KC237408) FS1
- ICMP 19563 (Sl) (KC237409) FS1
- ICMP 19513 (Sm) (KC237395) FS5
- ICMP 19519 (Sm) (KC237402) FS4
- ICMP 19512 (Sm) (KC237394) FS5
- ICMP 19545 (Sp) (KC237410) FS1
- ICMP 19546 (Sp) (KC237411) FS1
- ICMP 19547 (Sp) (KC237412) FS1
- M. ciceri UPM-Ca7 (U07934)
- ICMP 19518 (Sm) (KC237401) FS5
- ICMP 19551 (Sm) (KC237400) FS5
- ICMP 19517 (Sm) (KC237399) FS5
- ICMP 19516 (Sm) (KC237398) FS5
- ICMP 19515 (Sm) (KC237397) FS5
- ICMP 19514 (Sm) (KC237396) FS5
- M. shangrenense CCBAU 65327 (EU074203)
- M. australicum WSM2073 (NR102452)
- M. qingshengii CCBAU 33460 (NR109565)
- M. loti LMG 6125 (X67229)
- M. amorphae ACCC 19665 (AF041442)
- ICMP 19598 (Sl) (KC237338) FS1
- M. septentrionale SDW 014 (AF508207)
- B. elkani USDA 76 (U35000)
Fig. 2

*recA + glnII + rpoB*
Fig. 3

*nifH*