Genetic characterization of root-nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across south-eastern Australia

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Symbiotic relationships between legumes and nitrogen-fixing soil micro-organisms are of ecological importance in plant communities worldwide. For example, nutrient-poor Australian soils are often dominated by shrubby legumes (e.g. species of *Acacia*). However, relatively few studies have quantified patterns of diversity, host-specificity and effectiveness of these ecologically important plant–microbe interactions. In this study, 16S rRNA gene sequence and PCR-RFLP analyses were used to examine bacterial strains isolated from the root nodules of two widespread south-eastern Australian legumes, *Acacia salicina* and *Acacia stenophylla*, across nearly 60 sites. The results showed that there was extensive genetic diversity in microbial populations, including a broad range of novel genomic species. While previous studies have suggested that most native Australian legumes nodulate primarily with species of the genus *Bradyrhizobium*, our results indicate significant associations with members of other root-nodule-forming bacterial genera, including *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia*. Genetic analyses also revealed a diverse suite of non-nodulating bacterial endophytes, only a subset of which have been previously recorded. Although the ecological roles of these endosymbionts are not well understood, they may play both direct and indirect roles in promoting plant growth, nodulation and disease suppression.

INTRODUCTION

Legumes are one of the largest angiosperm groups and are broadly distributed across the globe (Polhill *et al.*, 1981). In many regions, native legumes are important contributors to ecological community structure and function, often supporting a broad range of insects (Bernhardt, 1989), providing food and habitat resources (Jackson, 2000; Sharpe & Goldingay, 1998; Thums *et al.*, 2005), and forming important symbiotic relationships with nitrogen-fixing soil micro-organisms (Sprent, 2001). Despite the importance of these associations in generating available nitrogen for legumes, as well as co-occurring non-leguminous species (Khanna, 1997), relatively few comparative studies of interactions involving native legumes and their symbionts exist, particularly on broad geographical scales (Weir *et al.*, 2004).

In Australia, shrubby legumes are a major component of almost all ecosystems (Maslin, 2001) and associations with nitrogen-fixing symbionts are particularly advantageous to plants growing in nutritionally poor Australian soils. As

with other regions of the world, Australia is now experiencing chronic soil and water degradation and a decline in biodiversity associated with broadscale vegetation clearing, necessitating prompt and cost-effective restoration. Legumes can play an important role in restoring degraded landscapes, particularly in regions where they are prolific or important components of the ecological community. Despite good evidence showing that introducing native rhizobia can improve restoration outcomes for legumes as well as associated species (Thrall *et al.*, 2005), little effort has been made to reintroduce appropriate and effective symbionts with their legume hosts simultaneously. This may be due partly to the paucity of information with respect to the diversity, distribution, abundance, host-specificity and effectiveness of these ecologically important plant–microbe interactions.

Known legume–symbiont associations involve both alpha- and betaproteobacteria (Moulin *et al.*, 2001). Alpha-proteobacteria involved in these interactions include species of the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, *Ensifer* (previously *Sinorhizobium* but now reinstated; <http://www.bacterio.cict.fr/e/ensifer.html>; Martens *et al.*, 2007), *Devosia*, *Phyllobacterium* and *Methylobacterium* (Chen *et al.*, 2001; de Lajudie *et al.*, 1998; Dreyfus *et al.*, 1988; Rivas *et al.*, 2002; Valverde *et al.*, 2005). Recent reports of betaproteobacterial

Abbreviation: CNI, close-neighbour interchange.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FJ687955–FJ688005 and FJ719310–FJ719366 as indicated in Table 1.

associations include species of the genera *Burkholderia* (Vandamme *et al.*, 2002), *Cupriavidus* (Sheu *et al.*, 2007), *Ralstonia* (Chen *et al.*, 2001; Verma *et al.*, 2004), *Ochrobactrum* (Trujillo *et al.*, 2005) and *Herbaspirillum* (Valverde *et al.*, 2003), although in the case of *Herbaspirillum* root nodulation has not been demonstrated. Here we report on the results of molecular characterization tests aimed at identifying bacterial strains associated with root nodules of two Australian tree legumes, *Acacia salicina* (Lindl.) and *Acacia stenophylla* (A. Cunn. ex Benth.), which are key restoration species in south-eastern Australia. These data form part of a long-term effort to evaluate geographical-scale patterns of genetic variation and adaptation in species of *Acacia* in Australia and their associated rhizobial bacteria. The overall goal is to better understand the interaction between plant–soil community viability and symbiont abundance, bacterial and host species genetics and remnant vegetation condition. From an applied perspective, a better understanding of these interactions and the implications this may have for the sourcing and deployment of a symbiont and host species germplasm will help to improve the cost-effectiveness of large-scale revegetation.

Previous studies of host–symbiont associations in temperate regions of south-eastern Australia indicated that the majority of root-nodule-forming bacteria isolated were of the genus *Bradyrhizobium* (Lafay & Burdon, 1998, 2001); however, some authors have suggested that, in drier regions, plant–microbe interactions are more likely to be dominated by faster-growing rhizobial genera such as *Rhizobium* (Barnet & Catt, 1991; Jenkins, 2003). *A. salicina* is widespread and common in the arid zone and reaches the drier regions in South Australia, Victoria and New South Wales, while *A. stenophylla* is widely distributed in inland arid areas from the north-east of Western Australia,

east through Northern Territory to Queensland and south to the Murray–Lachlan–Darling River system in New South Wales, Victoria and South Australia (Cowan, 1996). The primary goal of this study was to determine the number of rhizobial genospecies associated with each host species and the extent to which these were shared across hosts, to identify these genospecies to genus level and determine whether the genospecies genus affiliations were the same as those known to be present in other native Australian legumes.

METHODS

Bacterial isolation. Bacteria were isolated from soil collected from the rhizospheres of natural stands of *A. salicina* (28 sites) and *A. stenophylla* (30 sites) distributed across the Murray–Darling Basin in south-eastern Australia (Fig. 1); detailed descriptions of the study region, field sites and soil collection are given by Thrall *et al.*, (2007). It is possible that nodule-based samples from the field might better represent different components of rhizobial populations than those obtained from soil using seedlings. However, it should be noted that the same host species were used as were present at the sites as trap plants; furthermore, the symbiosis is likely to involve some degree of host choice (Simms *et al.*, 2006). Given the practical difficulty in collecting sufficient numbers of nodules from the roots of native leguminous trees (these species typically grow in very difficult soils such as clays), the widely used trapping approach was chosen as a good practical compromise.

Commercial quantities of seed for both host species were obtained from the Australian Seed Company (Hazelbrook, New South Wales). The seeds were surface sterilized in 100 % ethanol for 30 s, transferred to 4 % (w/v) sodium hypochlorite for 3 min, rinsed twice with sterile distilled water and scarified in concentrated sulphuric acid for 45–60 min. The seeds were rinsed 10 times, left at room temperature for several hours in the last wash and kept at 4 °C overnight. The seeds were then spread uniformly over water agar plates and incubated at 28 °C. When radicles reached ~3–4 mm, the germinated seeds were individually transferred to sterilized 200 ml glass test tubes containing

Fig. 1. Map (adapted with permission from Thrall *et al.*, 2007) showing site locations and identification numbers in New South Wales for the two study host species *A. salicina* (a) and *A. stenophylla* (b). Major rivers and streams within the Murray–Darling Basin (grey shaded area) are shown.

washed vermiculite with nitrogen-free seedling nutrient solution (Vincent, 1970).

Suspensions of soil from each site were made by mixing 90 ml sterile nitrogen-free nutrient solution with 10 g soil and shaking vigorously for 30 min. Each seedling (20–30 per site) was inoculated with 1 ml soil suspension. After 4–6 weeks of growth at 28 °C for 14 h (day) and 20 °C for 10 h (night), root nodules were collected, washed, surface sterilized in 70 % ethanol for 30 s and 4 % (w/v) sodium hypochlorite for 3 min and then rinsed five times. Nodules (one to two) from each seedling were individually crushed and an aliquot of the suspension was streaked onto yeast extract-mannitol agar plates, which were incubated at 28 °C for between 3 days and 2 weeks, depending upon bacterial growth rates. Well-separated single colonies (one per nodule) were transferred to fresh agar plates and subcultured two to three times until a pure culture was obtained.

DNA extraction and PCR amplification. DNA was extracted from each bacterial isolate as described by Chen & Kuo (1993) and quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen) and 10 ng μl^{-1} working solutions were prepared. Primers corresponding to nucleotide positions 8–28 (16S F27) and 1492–1509 (16S R1392) of the SSU rRNA sequence of *Escherichia coli* (Brosius *et al.*, 1978) were used to amplify DNA in 50 μl reactions containing 5 μl (50 ng) template DNA from each bacterial isolate, 25 pmol forward and reverse primers, 200 μM dNTPs (Astral Scientific), 2.5 U *Taq* DNA polymerase (New England BioLabs) and 1 $\times$ buffer (New England BioLabs). Reactions were performed in a Hybaid PCR Express thermocycler (Integrated Sciences) under the conditions of an initial cycle of 95 °C for 5 min, 55 °C for 2 min and 72 °C for 90 s followed by 30 cycles of 94 °C for 30 s, 55 °C for 60 s and 72 °C for 60 s with a final extension step of 72 °C for 5 min. Aliquots (8 μl) of each PCR product were digested with one of six restriction enzymes, *Hae*III, *Hinf*I, *Msp*I, *Mbo*II, *Rsa*I or *Taq*I (New England BioLabs), in a 40 μl digestion reaction for 3–4 h as specified by the manufacturer. The reaction volume was then reduced to 20 μl in a Jouan Rc1010 vacuum concentrator/centrifugal evaporator (Thermo Fisher Scientific). Restriction fragments were separated by gel electrophoresis on 2 % (w/v) agarose run at 180 V for 2 h, stained with ethidium bromide and photographed. Restriction profiles, based on the banding patterns of the six restriction enzymes, were determined for each isolate with each unique profile being considered as representing a distinct genomic species (genospecies).

16S rRNA gene sequencing. Genospecies that were identified by PCR–RFLP analysis were selected for 16S rRNA gene sequencing. PCR products were cleaned using the UltraClean DNA Purification kit (Mo Bio Laboratories) according to the manufacturer's instructions and forward and reverse sequences were determined using Big Dye terminator v3.1 (Applied Biosystems) cycle sequencing. Reactions (20 μl) were comprised of 4 μl Big Dye terminator v3.1 buffer (5 $\times$), 1 μl of either the 16S F27 or 16S R1392 primer (16 pmol), 2 μl Big Dye terminator v3.1 mix, 2 μl PCR product (50–60 ng) and 11 μl sterile distilled water. Reactions were amplified in a Hybaid PCR Express thermocycler (Integrated Sciences) under the following conditions: 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Reactions were precipitated with 80 μl fresh 75 % (v/v) 2-isopropanol at room temperature for 30 min and centrifuged at 14 000 r.p.m. for 30 min. The supernatant was removed, 150 μl 75 % (v/v) 2-isopropanol was added and the samples were centrifuged at 14 000 r.p.m. for 15 min. The 2-isopropanol was discarded and the samples were air-dried. The sequencing products were processed at the John Curtin School of Medical Research, Australian National University, using an ABI3730 capillary sequencer.

Sequence alignment, genospecies identification and phylogenetic analysis of nodulating bacteria. Sequences recovered

ranged from 800 to 1300 bp and were aligned using MEGA 4.1 and the multiple sequence alignment program CLUSTAL W (Tamura *et al.*, 2007). These were used to identify genospecies to the genus level by comparison with reference bacterial sequences in the Ribosomal Database Project II database (<http://rdp.cme.msu.edu/>). Further characterization was undertaken by comparing the genus affiliations with those listed on the ICSP Subcommittee on the Taxonomy of *Rhizobium* and *Agrobacterium* database (<http://edzna.ccg.unam.mx/rhizobial-taxonomy/>). These comparisons identified several isolates belonging to non-nodulating bacterial genera. The affiliations were confirmed by reinoculating these isolates onto three species of *Acacia* (*A. salicina*, *A. stenophylla* and *A. mearnsii*). For these inoculations, seedlings were grown in sterile vermiculite in 150 $\times$ 20 mm glass tubes closed with plugs of polyurethane foam and inoculated with 1 ml of a given culture (four replicate tubes per isolate). After 6 weeks of growth, seedlings were examined for the presence of nodules. DNA was extracted from any nodules formed to confirm isolate identity.

Phylogenetic analyses were conducted for nodulating bacterial genospecies with unique sequences between nucleotide positions 342 and 1349 of the *E. coli* 16S rRNA gene sequence (Brosius *et al.*, 1978). As a matter of interest, the genus *Herbaspirillum* was included in this analysis and, although this genus has not been shown to nodulate legumes, it has been isolated from legume nodules (Valverde *et al.*, 2003) and species of the genus are known to fix nitrogen endophytically in the roots of a range of leguminous and non-leguminous host plants (Schmid & Hartmann, 2003; Schmid *et al.*, 2006).

Three methods were used to infer evolutionary relationships using MEGA 4 (Tamura *et al.*, 2007). The minimum-evolution (ME; Rzhetsky & Nei, 1992) method was used to estimate evolutionary distances using the maximum-composite likelihood method (Tamura *et al.*, 2004) and the tree was searched using the close-neighbour interchange (CNI) algorithm (Rzhetsky & Nei, 1992). Evolutionary distances for neighbour-joining (NJ; Saitou & Nei, 1987) trees were computed using the maximum-composite likelihood method (Tamura *et al.*, 2004) while the maximum-parsimony tree (MP; Eck & Dayhoff, 1966) was obtained using the CNI algorithm (Nei & Kumar, 2000). Relationships among the bacterial isolates were assessed for all nodulating genospecies as well as for *Ensifer/Rhizobium* and *Bradyrhizobium* isolates separately. These latter analyses also included reference sequences of closely related species, as published by the ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium*, which were obtained from GenBank. *Devosia neptuniae* J1 (AF469072) was used as an outgroup reference (Fig. 3b). Maximum-parsimony was based on the CNI algorithm (Nei & Kumar, 2000). Bootstrap analysis for all trees was based on 1000 resamplings. No significant topological differences were found between the phylogenetic trees obtained by the different methods; thus, only neighbour-joining trees are presented here (Fig. 3). Given that the taxonomic placement of the genus *Agrobacterium* remains controversial (Farrand *et al.*, 2003; Young *et al.*, 2001), the name *Rhizobium* has been used rather than *Agrobacterium*. The genus name *Ensifer*, rather than the previous classification of *Sinorhizobium* (<http://www.bacterio.cict.fr/e/ensifer.html>), has been used for similar reasons.

RESULTS AND DISCUSSION

Genospecies diversity and dominance

A total of 1316 isolates from the 58 broadly distributed field sites were purified, characterized by restriction enzyme analysis and grouped into 119 previously unidentified genospecies. Of these genospecies, 108 (1285 isolates) could be reliably identified using 16S rRNA gene sequence

Table 1. Isolates and the genera that they are affiliated with based on 16S rRNA gene sequences

Genus affiliations were determined following a Ribosomal Database project II search. GenBank accession numbers of reference sequences are given in parentheses.

Genospecies	Genus	Closest species affiliation	Isolates (n)
Nodulating			
T41	<i>Rhizobium</i> (FJ688003)	<i>R. gallicum</i> S109 ^T (AY509211)	3
T3	<i>Rhizobium</i> (FJ719338)	<i>R. leguminosarum</i> (AF533683)	8
T24	<i>Rhizobium</i> (FJ719314)	<i>R. leguminosarum</i> (AF533683)	2
T35	<i>Rhizobium</i> (FJ687998)	<i>R. leguminosarum</i> (AF533683)	42
T42	<i>Rhizobium</i> (FJ688004)	<i>R. leguminosarum</i> DSM 30132 ^T (AY509899)	215
T44	<i>Rhizobium</i> (FJ719359)	<i>R. leguminosarum</i> (AF533683)	1
T45	<i>Rhizobium</i> (FJ719360)	<i>R. leguminosarum</i> DSM 30132 ^T (AY509899)	1
T49	<i>Rhizobium</i> (FJ688005)	<i>R. leguminosarum</i> (AF533683)	1
T67	<i>Rhizobium</i> (FJ719334)	<i>R. mongolense</i> (U89816)	8
T131	<i>Rhizobium</i> (FJ687993)	<i>R. mongolense</i> S152 ^T (AY509209)	3
T29	<i>Rhizobium</i> (FJ687996)	<i>R. rhizogenes</i> 163C ^T (AY206687)	7
T4	<i>Rhizobium</i> (FJ688001)	<i>Rhizobium</i> sp. MTR-3A ^T (DQ 898296)	1
T22	<i>Rhizobium</i> (FJ687995)	<i>Rhizobium</i> sp. CCBAU 41044 ^T (AY395697)	2
T30	<i>Rhizobium</i> (FJ687977)	<i>Rhizobium</i> sp. Aei-5 ^T (AF511492)	1
T34	<i>Rhizobium</i> (FJ687997)	<i>Rhizobium</i> sp. M16 ^T (AY177365)	1
T36	<i>Rhizobium</i> (FJ719345)	<i>Rhizobium</i> sp. P-26 ^T (AM411925)	1
T38	<i>Rhizobium</i> (FJ687999)	<i>Rhizobium</i> sp. rob20 ^T (AJ271902)	1
T39	<i>Rhizobium</i> (FJ688000)	<i>Rhizobium</i> sp. P-25 ^T (AM411924)	1
T40	<i>Rhizobium</i> (FJ688002)	<i>Rhizobium</i> genosp. R ^T (Z94803)	8
T120	<i>Rhizobium</i> (FJ687992)	<i>Rhizobium</i> sp. ORS 1411 ^T (AY500258)	1
T2	<i>Ensifer</i> (FJ687994)	<i>E. morelense</i> (AF452129)	1
T28	<i>Ensifer</i> (FJ719364)	<i>E. morelense</i> (AF452129)	1
T32	<i>Ensifer</i> (FJ687978)	<i>E. adhaerens</i> HAMB1 1631 ^T (AJ420774)	5
T33	<i>Ensifer</i> (FJ687979)	<i>E. adhaerens</i> LMG 20582 ^T (AJ420775)	1
T9	<i>Ensifer</i> (FJ687985)	<i>E. arboris</i> LMG 19223 ^T (AM181738)	5
T61	<i>Ensifer</i> (FJ687982)	<i>E. arboris</i> LMG 19223 ^T (AM181738)	2
T10	<i>Ensifer</i> (FJ687972)	<i>E. fredii</i> LMG 6217 ^T (X67231)	5
T12	<i>Ensifer</i> (FJ687974)	<i>E. fredii</i> LMG 6217 ^T (X67231)	119
T27	<i>Ensifer</i> (FJ687976)	<i>E. fredii</i> LMG 6217 ^T (X67231)	1
T5	<i>Ensifer</i> (FJ687980)	<i>E. meliloti</i> (AL591782)	22
T6	<i>Ensifer</i> (FJ687981)	<i>E. meliloti</i> (AL591782)	112
T7	<i>Ensifer</i> (FJ687983)	<i>E. meliloti</i> (AL591782)	7
T8	<i>Ensifer</i> (FJ687984)	<i>E. meliloti</i> (AL591782)	26
T11	<i>Ensifer</i> (FJ687973)	<i>E. meliloti</i> (AL591782)	1
T23	<i>Ensifer</i> (FJ687975)	<i>E. meliloti</i> SEMIA 103 ^T (AY904726)	1
T31	<i>Ensifer</i> (FJ719332)	<i>E. meliloti</i> (AL591782)	1
T93	<i>Bradyrhizobium</i> (FJ687966)	<i>B. elkanii</i> NA6605 ^T (AB072423)	1
T95	<i>Bradyrhizobium</i> (FJ687967)	<i>B. elkanii</i> NA6605 ^T (AB072423)	2
T96	<i>Bradyrhizobium</i> (FJ687968)	<i>B. elkanii</i> NA6605 ^T (AB072423)	3
T97	<i>Bradyrhizobium</i> (FJ719319)	<i>B. elkanii</i> NA6605 ^T (AB072423)	1
T98	<i>Bradyrhizobium</i> (FJ687969)	<i>B. elkanii</i> NA6605 ^T (AB072423)	4
T84	<i>Bradyrhizobium</i> (FJ687961)	<i>B. japonicum</i> YCK295 ^T (AB070566)	2
T86	<i>Bradyrhizobium</i> (FJ687963)	<i>B. japonicum</i> NA6086 ^T (AB072418)	1
T100	<i>Bradyrhizobium</i> (FJ719317)	<i>B. japonicum</i> NA6086 ^T (AB072418)	1
T101	<i>Bradyrhizobium</i> (FJ687955)	<i>B. japonicum</i> YCK295 ^T (AB070566)	1
T102	<i>Bradyrhizobium</i> (FJ687956)	<i>B. japonicum</i> NA6251 ^T (AB072414)	2
T103	<i>Bradyrhizobium</i> (FJ687957)	<i>B. japonicum</i> YCK295 ^T (AB070566)	1
T130	<i>Bradyrhizobium</i> (FJ687959)	<i>B. japonicum</i> YCK295 ^T (AB070566)	1
T85	<i>Bradyrhizobium</i> (FJ687962)	<i>B. liaoningense</i> LMG 18230 ^T (AJ250813)	3
T104	<i>Bradyrhizobium</i> (FJ687958)	<i>B. liaoningense</i> LMG 18230 ^T (AJ250813)	1
T90	<i>Bradyrhizobium</i> (FJ687964)	<i>Bradyrhizobium</i> genosp. B ^T (Z94812)	8
T91	<i>Bradyrhizobium</i> (FJ719361)	<i>Bradyrhizobium</i> genosp. B ^T (Z94812)	2
T43	<i>Bradyrhizobium</i> (FJ687960)	<i>Bradyrhizobium</i> genosp. B ^T (Z94812)	1

Table 1. cont.

Genospecies	Genus	Closest species affiliation	Isolates (n)
T92	<i>Bradyrhizobium</i> (FJ687965)	<i>Bradyrhizobium</i> sp. Pplu1-11 ^T (AF384140)	1
T13	<i>Mesorhizobium</i> (FJ719326)	<i>Mesorhizobium</i> sp. ML105 ^T (EU748910)	1
T14	<i>Mesorhizobium</i> (FJ687988)	<i>Mesorhizobium</i> sp. ML105 ^T (EU748910)	10
T20	<i>Mesorhizobium</i> (FJ687990)	<i>M. plurifarium</i> LMG 11892 ^T (Y14158)	1
T19	<i>Mesorhizobium</i> (FJ687989)	<i>Mesorhizobium</i> sp. Lo7-12 ^T (AF410897)	39
T65	<i>Achromobacter</i> (FJ719348)	<i>A. xylosoxidans</i> AU0665 ^T (AF411019)	2
T48	<i>Burkholderia</i> (FJ687970)	<i>Burkholderia</i> sp. LMG 19076 ^T (AF215704)	1
T110	<i>Burkholderia</i> (FJ719339)	<i>Burkholderia</i> sp. LMG 19076 ^T (AF215704)	1
T15	<i>Devosia</i> (FJ687971)	<i>D. neptuniae</i> J1 ^T (AF469072)	2
T50	<i>Phyllobacterium</i> (FJ687991)	<i>P. trifolii</i> (AY786080)	1
Non-nodulating			
T1	<i>Rhizobium</i> (FJ719340)	<i>R. radiobacter</i> Mushin 6 ^T (AJ389905)	1
T68	<i>Rhizobium</i> (FJ719335)	<i>R. radiobacter</i> NCPPB 1641 ^T (AJ389902)	1
T69	<i>Rhizobium</i> (FJ719322)	<i>R. radiobacter</i> NCPPB 1641 ^T (AJ389902)	13
T112	<i>Rhizobium</i> (FJ719313)	<i>R. radiobacter</i> CFBP 2712 ^T (AJ389886)	3
T115	<i>Rhizobium</i> (FJ719343)	<i>R. radiobacter</i> Mushin 6 ^T (AJ389905)	3
T117	<i>Rhizobium</i> (FJ719366)	<i>R. radiobacter</i> Mushin 6 ^T (AJ389905)	8
T118	<i>Rhizobium</i> (FJ719333)	<i>R. radiobacter</i> 0362 ^T (AJ389907)	1
T119	<i>Rhizobium</i> (FJ719352)	<i>R. radiobacter</i> CFBP 2712 ^T (AJ389886)	3
T122	<i>Rhizobium</i> (FJ719311)	<i>R. radiobacter</i> Mushin 6 ^T (AJ389905)	10
T51	<i>Herbaspirillum</i> (FJ719346)	<i>H. frisingense</i> GSF30 ^T (AJ238358)	5
T53	<i>Herbaspirillum</i> (FJ687986)	<i>H. frisingense</i> GSF30 ^T (AJ238358)	102
T57	<i>Herbaspirillum</i> (FJ687987)	<i>H. frisingense</i> GSF30 ^T (AJ238358)	2
T59	<i>Herbaspirillum</i> (FJ719318)	<i>H. frisingense</i> GSF30 ^T (AJ238358)	1
T78	<i>Herbaspirillum</i> (FJ719355)	<i>H. frisingense</i> GSF30 ^T (AJ238358)	1
T123	<i>Pseudomonas</i> (FJ719351)	<i>P. fluorescens</i> 2P24 ^T (AY447045)	1
T81	<i>Pseudomonas</i> (FJ719329)	<i>P. putida</i> (AB029257)	1
T75	<i>Pseudomonas</i> (FJ719312)	<i>Pseudomonas</i> sp. 3C_10 ^T (AY689036)	1
T77	<i>Pseudomonas</i> (FJ719357)	<i>Pseudomonas</i> sp. MFY82 ^T (AY331361)	385
T62	<i>Pseudomonas</i> (FJ719356)	<i>Pseudomonas</i> sp. MFY82 ^T (AY331361)	1
T79	<i>Paenibacillus</i> (FJ719315)	<i>P. agarexedens</i> DSM 1327 ^T (AJ345020)	1
T127	<i>Paenibacillus</i> (FJ719365)	<i>P. agarexedens</i> DSM 1327 ^T (AJ345020)	1
T56	<i>Paenibacillus</i> (FJ719337)	<i>P. amylolyticus</i> (AB073190)	1
T132	<i>Paenibacillus</i> (FJ719321)	<i>P. glycanilyticus</i> DS-1 ^T (AB042938)	1
T60	<i>Paenibacillus</i> (FJ719328)	<i>P. pabuli</i> (AB073191)	1
T72	<i>Bacillus</i> (FJ719316)	<i>B. fusiformis</i> DSM 2898 ^T (AJ310083)	2
T83	<i>Bacillus</i> (FJ719320)	<i>B. senegalensis</i> RS8 ^T (AF519468)	1
T73	<i>Brevibacillus</i> (FJ719350)	<i>B. brevis</i> (AB101593)	1
T74	<i>Brevibacillus</i> (FJ719331)	<i>B. brevis</i> (AB101593)	1
T47	<i>Caulobacter</i> (FJ719362)	<i>C. vibrioides</i> CB51 ^T (AJ009957)	1
T82	<i>Caulobacter</i> (FJ719327)	<i>C. vibrioides</i> CB51 ^T (AJ009957)	1
T128	<i>Enterobacter</i> (FJ719323)	<i>Enterobacteriaceae</i> sp. JM965 ^T (AJ302154)	1
T129	<i>Enterobacter</i> (FJ719310)	<i>Enterobacter</i> sp. M60-2 ^T (EF061896)	1
T105	<i>Microbacterium</i> (FJ719325)	<i>M. flavescens</i> (Y17232)	1
T107	<i>Microbacterium</i> (FJ719349)	<i>M. trichothecenolyticum</i> (AB004722)	1
T71	<i>Variovorax</i> (FJ719347)	<i>V. paradoxus</i> DSM 66 ^T (AJ420329)	1
T70	<i>Variovorax</i> (FJ719336)	<i>Variovorax</i> sp. MN 36.2 ^T (AJ313017)	2
T133	<i>Acinetobacter</i> (FJ719330)	<i>A. haemolyticus</i> (AF542962)	1
T46	<i>Ancylobacter</i> (FJ719324)	<i>Ancylobacter</i> sp. DSM 1107 ^T (AY211514)	1
T124	<i>Arthrobacter</i> (FJ719341)	<i>Arthrobacter</i> sp. Ellin146 ^T (AF408988)	1
T52	<i>Chitinophaga</i> (FJ719353)	<i>C. sancti</i> (M62795)	1
T108	<i>Comamonas</i> (FJ719342)	<i>C. testosteroni</i> Q10 ^T (AF519533)	1
T94	<i>Kaistia</i> (FJ719344)	<i>Kaistia</i> sp. WPCB167 (FJ006913)	1
T126	<i>Mycobacterium</i> (FJ719354)	<i>M. frederiksborgense</i> DSM 44346 ^T (AJ276274)	2
T109	<i>Roseateles</i> (FJ719358)	<i>R. depolymerans</i> 61B2 ^T (AB003625)	2
T116	<i>Stenotrophomonas</i> (FJ719363)	<i>S. maltophilia</i> LMG 10857 ^T (AJ131117)	1

analysis and were subsequently shown to include both alpha- and beta-proteobacteria from a broad range of nodulating (712 isolates, 55.5 %) and non-nodulating (573 isolates, 44.5 %) bacteria (Table 1). Of the 1285 isolates, 385 (~30 %) belonged to genospecies T77, which was identified as representing the non-nodulating genus *Pseudomonas*, while other genospecies with large numbers of isolates included T42 (*Rhizobium*; 215 isolates, ~17 %), T12 and T6 (*Ensifer*; 119 isolates, ~9 %, and 112 isolates, ~9 %, respectively) and T53 (*Herbaspirillum*; 102 isolates ~8 %). Most of the remaining isolates were extremely rare, often represented by a single isolate. The affiliations of nodulating isolates with specific genera in this study contrast with those observed in other studies conducted in Australia, in which only the genera *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium* were observed (Lafay & Burdon 1998, 2001; Marsudi *et al.*, 1999). This may reflect both the broader geographical scale sampled and the larger number of isolates included in this study compared with other studies, along with the fact that previous studies concentrated on temperate Australian legumes.

Of the 108 genospecies identified by gene sequence analysis, 68 were identified as nodulating bacteria and were found to be distributed mostly among nine genera: *Rhizobium* (20 genospecies), *Ensifer* (16), *Bradyrhizobium* (18), *Mesorhizobium* (4), *Burkholderia* (2), *Achromobacter* (1), *Devosia* (1) and *Phyllobacterium* (1). Several of the nodulating isolates identified had not been encountered previously in Australian soils. For example, genospecies T50 showed strong sequence similarity (100 %) with *Phyllobacterium trifolii* (AY786080), which was isolated from *Trifolium pratense* nodules in Spanish soils (Valverde *et al.*, 2005), and genospecies T15 was identified as a possible relative of *Devosia neptuniae*, isolated from the Indian aquatic legume *Neptunia natans* (Rivas *et al.*, 2002). Several beta-proteobacteria were also identified, including two genospecies (T53 and T57) with similarities to *Herbaspirillum lusitanum* P6-12^T (AF543312), found on *Phaseolus vulgaris* growing in north-eastern Portugal (Valverde *et al.*, 2003), and one genospecies (T480) with a strong similarity to *Burkholderia phymatum* STM815^T, found associated with *Machaerium lunatum* in French Guiana (Moulin *et al.*, 2001; Vandamme *et al.*, 2002).

The remaining 40, non-nodulating genospecies (~38 %) were found to belong to 19 diverse genera, the most common of which were represented by the taxa *Rhizobium radiobacter* (9 genospecies), *Pseudomonas* (5), *Herbaspirillum* (5) and *Paenibacillus* (5). This large and diverse component composed of non-nodulating bacteria is consistent with previous studies that have reported the identification of a range of endosymbiotic but non-nitrogen-fixing bacteria (commonly including species of the genus *Pseudomonas*) from root nodules (Benhizia *et al.* 2004; Li *et al.* 2008; Muresu *et al.*, 2008; Sturz *et al.*, 1997; Zakhia *et al.*, 2006). While many of the non-nodulating bacteria identified in this study (e.g. *Pseudomonas fluorescens*) have been previously reported as common endosymbionts of root nodules

(Muresu *et al.*, 2008; Zakhia *et al.*, 2006), several isolates showed strong affiliations with bacterial genera that have not previously been recorded as being endophytic (e.g. *Caulobacter*, *Variovorax*). The roles of non-rhizobial endosymbionts in environmental systems have not been well-studied but it has been reported that both nodulation and legume nitrogen fixation can be enhanced by co-inoculation with endophytic plant-growth-promoting bacteria. Both *Bacillus subtilis* and *Bacillus thuringiensis* have been isolated from vigorous field-grown soybean plants and co-inoculating soybean plants with *Bacillus thuringiensis* and *Bradyrhizobium japonicum* has produced consistent increases in yield (Bai *et al.*, 2003). Reports also suggest that some endophytes produce metabolites that suppress the growth of plant pathogens (Andrews & Harris, 2000).

Phylogenetic relationships among isolates

Comparative analysis of 16S rRNA gene sequences remains an important tool for taxonomic identification in bacteria (Stackebrandt *et al.*, 2002); however, relationships can vary depending upon the location and length of the sequence analysed (*Rhizobium galegae*; Martinez-Romero & Caballero-Mellado, 1996; Nour *et al.*, 1994; Willems & Collins, 1993) (*Rhizobium etli*; Martinez-Romero & Caballero-Mellado, 1996). The bacterial 16S rRNA gene sequence has nine hypervariable regions (Chakravorty *et al.*, 2007), six of which were covered by the 1000 bp sequence length in our analysis, and provided a resolution similar to that observed in other studies (Kwon *et al.*, 2005). The 52 nodulating genospecies identified in this study fell into six major groups and were affiliated mostly with existing genera (Fig. 2). *Ensifer* and *Rhizobium* isolates formed two discrete groups at the top of the dendrogram with the exception of the *Ensifer* isolate T2, which was included in the *Rhizobium* group. Interestingly, *Phyllobacterium* and *Mesorhizobium* isolates were placed between *Ensifer* isolates T32 and T33 and the major *Ensifer* group. The single *Devosia* isolate (T15) fell between *Ensifer* and *Rhizobium* groups and above the *Bradyrhizobium* cluster. The remaining isolates including the *Achromobacter*, *Burkholderia* and *Herbaspirillum* isolates grouped together at the bottom of the dendrogram.

Phylogenetic relationships within the *Ensifer*/*Rhizobium* and *Bradyrhizobium* groups

The comparison of the *Rhizobium* and *Ensifer* genospecies, in conjunction with 26 reference sequences, partitioned the data into two major groups (Fig. 3a). Group A was primarily composed of *Ensifer* reference sequences, but also included *Rhizobium giardinii*. In this group, T34 was most closely aligned with *R. giardinii* CCBAU 85040^T (97 % bootstrap support, 100 % sequence identity), while T10 had strong sequence identity with *Ensifer saheli* LMG 11864^T (99 % sequence identity) but only with moderate bootstrap support (55 %). Group B was mostly comprised of *Rhizobium* sequences with several genospecies showing strong alignment with known reference sequences. For

Fig. 2. Phylogenetic relationships among nodulating genospecies inferred from 16S rRNA gene sequences using the neighbour-joining method. GenBank accession numbers for the 16S rRNA gene sequences of the novel isolates are listed in Table 1. Host species are indicated in parentheses: Sa, *Acacia salicina*; St, *Acacia stenophylla*. Bootstrap values >50%, based on 1000 replications, are shown at branching points. Bar, 2% sequence divergence.

example, T120 was strongly aligned with *Rhizobium huautilense* S02^T (AF025852) (99% sequence identity, 91% bootstrap support), while T29 was strongly aligned with *Rhizobium lusitanum* CCBAU 03301^T (99% sequence identity, 73% bootstrap support). Interestingly, rhizobial genospecies Q, which is presumed to be widely distributed, having been isolated from 11 legume hosts including three species of *Acacia* from 23 sites (Lafay & Burdon, 2001), was not detected in this study. However, T29 did show some sequence similarity with Q^T and was isolated from seven broadly distributed sites. Resolution was poor among the 13 *Bradyrhizobium* reference sequences and 15 genospecies (Fig. 3b), with the two groups differentiating along similar lines to those observed among Korean isolates from a range of leguminous hosts (Kwon *et al.*, 2005). Here, the *Bradyrhizobium elkanii* reference strains, the *Bradyrhizobium* genospecies P and four of the genospecies from this study (group B) were separated from the remaining sequences, which were from *Bradyrhizobium japonicum*, *Bradyrhizobium canariense*, *Bradyrhizobium* spp., the *Bradyrhizobium* genospecies A, D, F, B, I, O (Lafay & Burdon, 1998) and the remainder of the genospecies identified in this study (none of which were closely associated with any identified by Lafay & Burdon, 1998; 2001).

Due to the small number of isolates associated with the remaining genera, phylogenetic analysis was not undertaken. However, sequence comparisons with reference strains indicated that genospecies T19 was closely aligned (100% sequence identity) with *Mesorhizobium* sp. Ellin189, previously isolated from soil collected in Victoria, Australia (Schoenborn *et al.*, 2004), and genospecies T20 had a very high sequence identity (100%) to *Mesorhizobium plurifarium* LMG 7854^T, previously isolated from *Acacia laeta* in Senegal (de Lajudie *et al.*, 1994). In contrast, genospecies T14 had low sequence identity with all of the reference sequences. Genospecies T50 was strongly aligned with *Phyllobacterium trifolii* PETP02^T (AY786080) (100% sequence identity), while T53 and T57 were very similar to each other (98% sequence identity). Genospecies T48 was most closely aligned with *Burkholderia phymatum* STM815^T (97% sequence identity) while T15 was most closely associated with *Devosia neptuniae* J1^T (95% sequence identity).

Host-genospecies affiliations

While more specific patterns of genospecies identity, abundance and distribution across the study region are currently being explored, some interesting host-symbiont associations were apparent. For example, six of the

Fig. 3. Phylogenetic relationships among nodulating genospecies of the genera *Rhizobium* and *Ensifer* (a) and among genospecies of the genera *Bradyrhizobium* (b) inferred from 16S rRNA gene sequences using the neighbour-joining method. GenBank accession numbers for the 16S rRNA gene sequences of the novel isolates are listed in Table 1. Host species of *Acacia* are indicated in parentheses (Sa, *A. salicina*; St, *A. stenophylla*). Bootstrap values >50 % based on 1000 replications are shown at branching points. Bars, 2 % (a) and 0.2 % (b) sequence divergence.

Rhizobium isolates (Fig. 2) were exclusive to *A. salicina*, three were isolated only from *A. stenophylla* and the remaining six were present on both species. Two *Ensifer*

isolates were only found on *A. salicina*, whereas six were isolated from *A. stenophylla*. In contrast to the apparent lack of host preference with regard to the *Rhizobium* and

Ensifer isolates, most *Bradyrhizobium* isolates were found on *A. salicina* only, with one, T90, isolated from both acacias and T102 present on *A. stenophylla* only. This finding may have important implications for the successful re-establishment of legume–symbiont associations across south-eastern Australia. Only three *Mesorhizobium* isolates were identified. Both genospecies T19 and T14 were abundant and isolated from both species of *Acacia*, whereas T20 was relatively rare and only found on *A. salicina*. Two *Mesorhizobium* isolates were detected by Lafay & Burdon (1998) across 32 legume host species representing multiple genera, including genospecies T, isolated only once from *Acacia obliquinervia*, and genospecies S^T, isolated only once from *Goodia lotifolia*. Interestingly, Weir *et al.* (2004) found that native New Zealand legumes were only nodulated by *Mesorhizobium*, whereas introduced legumes were nodulated by *Bradyrhizobium*.

Conclusions

The unexpectedly diverse array of both nodule-forming and endosymbiotic bacteria observed in these two acacia hosts is an indication of how little is known about the diversity, distribution, abundance and effectiveness of legume–symbiont interactions. For example, this study has shown that quite different rhizobial genera may predominate across more broad geographical scales, encompassing semi-arid and arid regions, than previously shown in studies of temperate sites (Lafay & Burdon 1998, 2001). More locally, previous studies have also shown that rhizobial community composition and symbiotic effectiveness varies with regard to soil salinity (Thrall *et al.*, 2009). Clearly, further studies are needed to better quantify how rhizobial community structure and function evolve in relation to environmental gradients and host community composition. Understanding these relationships, as well as the ecological roles that other endophytic microbes play, is likely to improve both the cost-effectiveness and the success of the reintroduction of legume species in highly degraded landscapes throughout the world.

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