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Nitrogen fixing bacterial communities in invasive legume nodules and associated soils are similar across introduced and native range populations in Australia

Running head: Bacterial communities of legumes in Australia

KEYWORDS: *Acacia*; Australia; free-living nitrogen fixers; invasion; legumes; mutualism; rhizobia

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ABSTRACT

Aim

Understanding interactions between invasive legumes and soil biota in both native and introduced ranges could assist in managing biological invasions. We analysed the diversity of putative nitrogen fixing bacteria (i.e., *nifH* gene present) associated with five invasive legumes, four *Acacia* spp. and a sister taxon *Paraserianthes lophantha* in introduced and native range populations in Australia. We predicted that, because these host species are widely distributed, they are likely to encounter different nitrogen-fixing bacterial communities in soils and nodules across their introduced and native ranges.

Location

Australia.

Methods

*NifH* genes were amplified from rhizosphere soils collected from beneath each species (multiple populations) within their native and introduced range and directly from nodules collected from plants previously grown in the glasshouse using field-collected soil as inoculum. *NifH* gene sequences from soils and nodules were 454 pyrosequenced and assigned to taxonomic groups based on *nifH* consensus taxonomy.

Results
We found no difference in the NFB community of soils or nodules between native and introduced ranges across the five species, suggesting that these legumes encounter similar NFB communities in soils across Australia. *Bradyrhizobium* was the most abundant rhizobial genus present in both soils and nodules. *Bradyrhizobium* species found in nodules were significantly different across the ranges for *A. longifolia*.

**Main conclusions**

The results indicate that these invasive legumes have similar nitrogen fixing bacterial communities in their rhizosphere and nodules across Australia, with the exception of *A. longifolia*. This species has diverse *Bradyrhizobium* genotypes in its nodules suggesting that *A. longifolia* may be a more generalist host compared to the other four legumes. Thus it is unlikely that the invasive success of these legumes is constrained by the absence of suitable bacterial symbionts in soil. Better knowledge of legume-soil interactions could facilitate more informed and effective management of invasive legumes in their introduced ranges in Australia and elsewhere.
Introduction

Soil microbes are increasingly acknowledged to play significant roles in invasion outcomes for many plant species (Inderjit & Cahill, 2015; Vestergård et al., 2015). For example, it has been suggested that the spread of plant invaders may depend on the successful establishment of key mutualisms in new ranges (Simberloff & Von Holle, 1999; Richardson et al., 2000). This may be particularly true for plants which have specific inter-dependencies with symbiotic mutualists such as legumes and nitrogen-fixing bacteria.

Woody legumes, especially Acacia species, are considered to be some of the worst invaders globally (Richardson & Rejmánek, 2011). The invasion success of acacias has been largely credited to their extensive use globally in agro-forestry and horticulture (Griffin et al., 2011). This widespread use of acacias opened a path to the colonization of novel communities beyond their natural range and has resulted in significant impacts on invaded ecosystems via induced changes to soil chemistry and microbial assemblages (Marchante et al., 2008; Le Maitre et al., 2011).

Generally, it has been proposed that invaders can be either constrained by mutualists in the absence of suitable partners (Parker, 2001) or benefit from newly acquired symbionts in novel ranges (Marler et al., 1999; Parker et al., 2007). There is certainly evidence that lack of compatible key soil mutualists serves as a constraint for successful establishment in the novel range for some species (Díez, 2005; Nuñez et al., 2009; Dickie et al., 2010). Legumes have been reported to rely extensively on mutualisms (e.g., rhizobia, mycorrhizal fungi) to successfully colonize and establish in novel areas (Sprent & Parsons, 2000; Parker, 2001). Absence or low densities of compatible rhizobia have been shown to limit range expansion and fitness of some
legume species (Stanton-Geddes & Anderson, 2011), including invasive Australian acacias in New Zealand (Wandrag et al., 2013). Broad symbiotic promiscuity and ability to nodulate at low rhizobial abundance have been described as significant advantages for invading legumes (Parker, 2001; Perez-Fernández & Lamont, 2003; Rodríguez-Echeverria et al., 2011). Previous studies have shown that some invasive woody legumes are able to readily nodulate (Lafay & Burdon, 2006) and associate with novel bacterial communities in their exotic ranges (Marsudi et al., 1999; Amrani et al., 2010; Callaway et al., 2011; Ndlovu et al., 2013).

Despite the evidence for promiscuity for some host species, there are also reports showing that some invasive legumes in their invasive range have specificity towards rhizobia from their native range (Chen et al., 2005). Such results were reported for A. longifolia in Portugal which was found to associate with rhizobial communities that were very similar to those from A. longifolia's native range in south-east Australia (Rodríguez-Echeverria, 2010). Thus, there appears to be considerable variation between legume hosts and their symbiotic associations across introduced and native ranges.

There is evidence to suggest that invasive acacias (sensu Richardson et al. 2011) could be more promiscuous, i.e., they are able to associate with more diverse rhizobia, than non-invasive acacias (Klock et al., 2015). However, there is also evidence to suggest that some acacias (e.g., A. cyclops, A. pycnantha) are able to non-specifically nodulate with both fast- and slow-growing rhizobia in their novel ranges (Marsudi et al., 1999; Mohamed et al., 2000; Lafay & Burdon, 2006; Ndlovu et al., 2013). Thus, quantification of the role of mutualists, such as rhizobia, in determining invasion
success could enhance our understanding of species’ invasion potential more generally.

In addition to known rhizobia, legume nodules harbour many other endophytes (Hoque et al., 2011; De Meyer et al., 2015), whose role within plants is largely unknown. Some authors have suggested that endophytes constitute an important component of the nodule bacterial community (Velázquez et al., 2013) and perform important functions such as assisting the host plant in pathogen control (El-Tarabily et al., 2010) and growth promotion (Ibáñez et al., 2009). To better predict the role of bacterial communities in plant invasions, we need to understand the diversity and function of nodule endophytes.

The primary aim of this study was to determine whether invasive Australian legumes [i.e., A. cyclops, A. longifolia, A. melanoxylon, A. saligna and Paraserianthes lophantha (hereafter collectively termed legumes)] encounter and associate with different nitrogen-fixing bacterial (NFB) communities in soils and nodules in their introduced range compared to native Australian range. We hypothesized that, because these host species are widely distributed and considered invasive, they are likely to accumulate different NFB nodule communities across introduced and native range populations.

Materials and methods

Study species

Four Acacia species (A. cyclops A.Cunn. ex G.Don, A. saligna (Labill.) H.L. Wendl, A. longifolia (Andrews) Willd. and A. melanoxylon R.Br.), and a close relative, P. lophantha (Willd.) I.C. Nielsen, were chosen as host species. Acacia cyclops, A. saligna and P. lophantha are native to Western Australia, but have been introduced to
the eastern Australian states (New South Wales, Victoria and South Australia) where they have naturalised and become invasive. *Acacia longifolia* and *A. melanoxylon* are native to south-east Australia, but have been introduced to and become invasive in Western Australia.

**Study sites and soil sampling**

To characterise the diversity of organisms putatively capable of N fixation and associated with our study species, we collected soil samples in December 2009 within the native and introduced ranges of each of the five species, across south-eastern and south-western Australia (Fig. 1 and details in Supporting Information Appendix S1).

For each species, with the exception of *A. melanoxylon* for which we only had four populations in its introduced range, we sampled soil under five trees from each of five populations within each range [5 species x 2 ranges (native and introduced) x 5 populations]. Thus, we sampled five populations in the native and introduced ranges (n=5) for most species except *A. melanoxylon* (n=5 to 4, respectively). A total of 1000 g of soil was collected beneath each tree, as close to roots as possible, at a depth of 10-15 cm and then bulked for each population. Soils were kept in a cooler in the field before being stored at 4°C. Soils were subsampled for genetic analysis within a week.

Soils were sieved to 2 mm to remove leaves and other coarse material and to homogenise samples. Soil subsamples were placed in sterilized falcon tubes and stored in the freezer at -20°C until further analysis. All sampling and processing equipment, including sieves, were sterilized with 90% ethanol between populations.

**Nodule collection**
To assess putative nitrogen fixer diversity in nodules we extracted DNA from nodules, collected from plant roots as part of a previous glasshouse experiment designed to evaluate the role of soil microbial communities (predominantly rhizobia) in determining cross-continental invasion success of the same five woody legume species used in this study (see Birnbaum et al. 2012 for details). Depending on seed availability, we had for *A. cyclops* n=4 (native) and n=3 (introduced), for *A. longifolia* n=3 and n=4, for *A. melanoxylon* n=3 and n=2, for *A. saligna* n=2 and n=3 and for *P. lophantha* n=2 and n=3 populations, respectively. The plants in that experiment were grown in field collected soils and assessed for growth in both introduced and native range soils. From each plant 2-5 nodules were collected and surface sterilized with 90% ethanol and distilled water before being stored at -20°C in a plastic jar filled with silica beads and cotton wool (Somasegaran, 1994). Nodules from 10 replicate plants from each of 29 population/range soil combinations were pooled for DNA extraction. Nodules (0.05 g) were crushed in liquid nitrogen to create a homogenised sample for DNA extraction.

**Molecular analysis**

DNA from 49 soil and 29 nodule samples was isolated using a PowerSoil and PowerPlant DNA isolation kit, respectively, following the manufacturer’s protocol (MO Bio Laboratories, Inc. Carlsbad, CA). *NifH* was amplified from soils and nodule DNA using a nested PCR with the internal primer pair *nifH* 1 (5’-TGY GAYCCN AAR GCN GA-3’) and *nifH* 2 (5’-ADN GCC ATC ATY TCN C-3’) (Zehr & McReynolds, 1989) and the external primers *nifH* 3 (5’-ATR TTR TTN GCC ATC ATY TCN C-3’) and *nifH* 4 (5’-TTY TAY GGN AAR GGN GG-3’). DNA was amplified using the HotStarTaq Plus Master Kit (Qiagen, Valencia, CA) for PCR under
following conditions: 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds; 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes (Dowd et al., 2008). Second PCR step was performed for 454 amplicon sequencing under the same conditions and primer set described above. Following second PCR, all amplicon products from different samples were mixed in equal volumes, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) (Dowd et al., 2008). DNA fragments' size and concentration were measured by using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner Biosystems, CA, USA) before sequencing commenced using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to determine the bacterial communities present according to the protocol in Dowd et al. (2008). More details for methods used in the molecular analysis for PCR amplifications and sequencing that were performed by the Research and Testing Laboratory (Lubbock, Texas, USA) can be found in protocols described in Smith et al. (2010) and Dowd et al. (2008).

**Bioinformatic analyses**

Downstream sequence analyses were performed on nifH sequences from nodules and soils using MOTHUR 1.22.0 (Schloss et al., 2009) following the adapted sequence quality-control pipeline analysis described in detail in Schloss et al. (2011), until chimeric sequences were removed. Sequences were included in subsequent analyses only if they carried the correct primer sequence and were ≥150 bp and ≤400 bp long. Following the pipeline analysis, sequences were blasted with a BLAST e-value > 0.001 against an existing nifH database that has 16 989 nifH sequences (Gaby &
Only the sequences that returned a blast hit of sequence identity ≥ 90% were kept and used for further downstream analysis. Approximately 13% of sequences did not match any existing sequence in the database and thus were removed from further analysis. Remaining sequences were checked for chimeras and a further 2% were removed from the dataset. After checking for chimeric sequences nodule and soil nifH sequences were split into separate groups (nodules and soils) and pre-clustered at 1% within each group. Following pre-clustering, nodule and soil sequences were classified using nifH consensus taxonomy (Gaby & Buckley, 2011) with the consensus confidence threshold of 51%. Furthermore, OTUs (Operational Taxonomic Unit) containing only a single sequence (i.e. singletons) were omitted from the analysis as they are likely to result from pyrosequencing errors (Tedersoo et al., 2010). The resultant OTU x sample matrix was binary transformed to presence/absence and used for subsequent analyses to identify differences in NFB at genus level. Additionally, to further explore the diversity of the dominant genus known to symbiotically fix N₂ and nodulate with legumes (Bradyrhizobium) sequences belonging to this genus were clustered at 97% sequence identity and resultant OTU’s analysed separately.

Statistical analyses

Principal coordinates analysis (PCA) based on a Bray-Curtis dissimilarity matrix was carried out on presence/absence transformed data generated from the OTU species matrix for soil and nodule NFB data separately. This allowed visual inspection to identify differences between introduced and native range populations for all host species. We used binary data to circumvent potential problems associated with
inferring abundances from amplion data and those associated with using proportional
data (relative abundance) (Amend et al., 2010).

Upon inspection of PCA results, permutational multivariate analysis of variance
(Permanova) (Anderson, 2001) was used (9999 permutations) to test for differences in
microbial community composition among species, range (native versus introduced)
and location (south-east versus south-west) as well as their interactions. This analysis
was done separately for soil and nodule matrices. Mantel tests (Mantel, 1967; Mantel & Valand, 1970) were performed to compare soil
and nodule dissimilarity matrices in order to determine if there was a correlation
between soil and nodule community composition. Additionally, the relative
abundance of NFB OTUs in soil and nodule data was calculated for each host species
and population. To estimate richness across host ranges, we used rarefaction analysis
to produce taxon accumulation curves using 1000 randomizations without
replacement. To estimate diversity among ranges, we calculated Shannon diversity
index. This metric allowed us to measure the OTU richness in a population of plants,
and compare the overall richness in populations between native and introduced ranges
per species.

PCA analysis and ordination, Mantel test and Shannon diversity were performed in
the R 3.1.1 programming language (R Core Team, 2015) using ‘vegan’ (Oksanen et
al., 2011) and ‘BiodiversityR’ (Kindt & Coe, 2005) packages. Permanova analysis
was performed in PRIMER 6. Rarefaction analysis was conducted in MOTHUR 1.22.0.

**Results**

**Nitrogen fixing bacterial communities in soils**
A total of 119 NFB genera (from 358,349 sequences) were identified from soil collected from the rhizospheres of the studied species, across both native and introduced ranges. Of these, 24 genera (13.5% or 48,543 sequences) had no known bacterial classification. Seven genera (13.3% or 47,779 sequences) are known to nodulate and form symbiotic relationships with legumes (Weir, 2016). Of these seven NFB genera, an overwhelming number of sequences (i.e. 94% or 45,095) belonged to *Bradyrhizobium*. The majority of detected taxa in soil, i.e. 112 NFB genera (including the unclassified sequences), belonged to genera not known to be rhizobial symbionts.

The overall Permanova model for soil NFB community composition at genus level revealed a significant interaction between native range soils and host species, suggesting that soil NFB composition is driven by larger geographic-scale variation between the NFB communities of south-eastern and south-western Australia (Table 1).

Rarefaction analysis showed soil NFB taxon accumulation curves reached asymptotes in all cases, indicating that sampling effort captured the majority of NFB diversity in these samples (Fig. 2). Overall bacterial OTU richness at genus level was not significantly different in soils across either host species or ranges (Appendix S1).

Pairwise analyses between *A. cyclops* and the other four host species [i.e., species x location (south-east versus south-west)] showed that soil NFB composition at the genus level for *A. cyclops* differed significantly from those of *A. melanoxylon*, *A. saligna* and *P. lophantha* (Table 2). Overall, no differences in soil NFB composition were detected within host species across their introduced and native ranges (Appendix S2 Figure S1), suggesting that soil NFB composition is similar across these geographically disjunct areas for these species, at least at the genus level.
The four most common and abundant soil bacterial genera (see Fig. 3) identified from nifH sequences belonged to organisms that are not known to be legume symbionts. Among the most common soil taxa that are classified as rhizobia (i.e., nitrogen fixing and nodulating bacteria) (Weir, 2016) found from nifH sequences (Fig. 3), there were seven OTUs belonging to the following genera: Aminobacter, Azorhizobium, Bradyrhizobium, Burkholderia, Ensifer, Methylobacterium and Phyllobacterium.

*Bradyrhizobium* is not listed in the Weir (2012) database as rhizobia. However there is one report showing this genus could contain legume-nodulating members, and it is closely related to another known legume-nodulating genus *Mesorhizobium* (Maynaud *et al.*, 2012).

Because *Bradyrhizobium* was the most abundant confirmed rhizobial genus, we subsequently analysed whether there was variation within this genus across ranges, locations and host species. Results revealed that host species and location indeed had a significant effect on *Bradyrhizobium* composition in the soil (Table 1), which was driven by pair-wise differences between *A. cyclops* and the other four host species (Table 3a). Differences in *Bradyrhizobium* composition across introduced and native ranges was close to significant (α=0.05) only for one host species (*A. longifolia*, \( t_{1.8} = 1.41, P = 0.082 \)) out of the five studied legumes.

### Nitrogen fixing bacterial communities in nodules

At genus level, a total of 29 genera (261,438 sequences) were classified as NFB originating from legume nodules. Of these, six genera (38.9% or 101,952 sequences) had no known bacterial classification. Five genera (34.7% or 90,906 sequences) were previously known to nodulate and form symbiotic relationships with legumes: *Aminobacter, Azorhizobium, Bradyrhizobium, Ensifer and Phyllobacterium* (Weir,
Burkholderia and Methylobacterium were found in the soils, but were not detected in the nodules. Of these five NFB genera, an overwhelming number of sequences (i.e., 31% or 82,165) belonged to Aminobacter. Bradyrhizobium was the second most common known rhizobial genus with 7,234 sequences (2.7%). Similar to soils, the majority of detected taxa in nodules (i.e., 24 genera, including the unclassified sequences) belonged to genera not known as rhizobia (65.2% or 170,532 sequences).

The overall Permanova model for nodule NFB community composition did not reveal any variation in NFB composition across host species and ranges at the genus level suggesting that, broadly speaking, these five legumes contained similar pools of NFB taxa in their nodules across introduced and native ranges (Table 1, Appendix S2 Figure S2). Rarefaction analysis showed that nodule NFB taxon accumulation curves reached asymptotes in all cases, indicating that sampling effort captured the majority of NFB taxa in these samples (Fig. 4). Overall bacterial OTU richness was not significantly different across host species or ranges at the genus level (Appendix S1).

The five most abundant genera in nodules were similar to the five most abundant NFB in soils (Fig. 5). Furthermore, all the taxa found in nodules were also found in soils, and we found a significant correlation between the two matrices (i.e., nodule and soil matrices) using the Pearson’s product-moment correlation with 999 permutations (Mantel statistic $r = 0.2347, P = 0.026$).

Similarly to soils, Bradyrhizobium was the most abundant confirmed rhizobial genus in the nodules. Permanova analysis of variation within Bradyrhizobium revealed that species and location interaction had a significant effect on nodule Bradyrhizobium spp. composition (Table 1), which was driven by pair-wise differences between A. cyclops and the other four host species (Table 3b). Additionally, Bradyrhizobium spp.
composition across introduced and native ranges was significantly different for one host species *A. longifolia* \( (t_{1,5} = 2.12, P = 0.024) \) and close to significance for *P. lophantha* \( (t_{1,3} = 2.09, P = 0.058) \).

**Discussion**

**Nitrogen fixing bacterial communities in soils**

Overall, at genus level, we found that host species by range had a significant interactive effect on soil NFB composition. Pairwise analyses between all hosts and ranges (native versus introduced) revealed that this effect was driven solely by one host, *A. cyclops*, whose soil NFB communities predominantly from the introduced ranges (i.e., Yorke Peninsula in South Australia) were significantly different from those of *A. melanoxylon* (native range, south-east), *A. saligna* (native range, south-west) and *P. lophantha* (native range, south-west). Notably, in our previous study, we found that *A. cyclops*’ soil fungal communities were also different from those of the other four host species as well as between its own introduced and native range populations (Birnbaum *et al.*, 2014). Thus it is plausible that *A. cyclops* has inherently different soil microbial communities in its rhizosphere compared to the other four host species.

We are not aware of other studies describing soil fungal or NFB communities of *A. cyclops*. Limited published evidence on *A. cyclops* and its rhizobial communities from nodules suggests that this species is non-specifically nodulated by both fast- and slow growing rhizobia in its introduced range in Libya (Mohamed *et al.*, 2000). Estimates of rhizobial abundance from introduced and native range soils in Australia have also shown no differences between ranges (Birnbaum *et al.*, 2012) suggesting that *A.
cyclops is not constrained by the absence of rhizobia and nodulates with diverse rhizobia which may have contributed to its invasion success. In terms of known nitrogen fixing and nodulating bacteria, we found three genera (e.g. Bradyrhizobium, Ensifer (formerly Sinorhizobium) and Azorhizobium) to be among the abundant groups in our soil samples. Bradyrhizobium has been frequently reported to be one of the most common rhizobial genera in the nodules of Acacia spp. in Australia (Stępkowski et al., 2012), particularly in the south-eastern (Burdon et al., 1999; Lafay & Burdon, 2001) and south-western (Marsudi et al., 1999) regions, as well as in introduced ranges in Portugal (Crisostomo et al., 2013) and South Africa (Ndlovu et al., 2013). Thus, it is not surprising that it was found in the soils of all species across introduced and native populations. Ensifer, on the other hand, was found only in two western natives’ (i.e., A. cyclops and P. lophantha) introduced populations in the south-east, and was absent from native and introduced population soils of A. longifolia and A. melanoxylon. Both Bradyrhizobium and Ensifer co-occurred only in one introduced A. cyclops population, both in soils and nodules. Although less common than Bradyrhizobium, Ensifer has been previously found in the nodules of some Acacia spp. in south-eastern Australia (Hoque et al., 2011) as well as in introduced ranges in several African countries (Räsänen et al., 2001; Amrani et al., 2010). Azorhizobium was the least common known rhizobial taxa found in the soils of these legumes, and it has been linked more often to other leguminous host species than acacias (Dreyfus et al., 1988; Boivin et al., 1997). In general, we found no statistically significant differences in soil NFB communities across introduced and native ranges within the five target plant species, suggesting that these legumes encounter similar NFB communities in soils across the Australian continent. Thus, it is unlikely that the absence of compatible bacterial communities
has constrained these species’ naturalisation and invasion success in novel environments across Australia. This is further evidenced by our previous findings that most of these invasive legumes perform (based on biomass data) equally well in both introduced and native range soils (Birnbaum & Leishman, 2013).

Nitrogen fixing bacterial communities in nodules

Overall, across our studied host species, we found no differences in NFB communities at the genus level in nodules or between native and introduced ranges. In accordance with the results from our analyses of associated soil communities, *Bradyrhizobium* was the most common known rhizobial genus occurring in all species’ introduced and native range nodules, although considerably less *Bradyrhizobium* was detected from *A. cyclops* nodules across both ranges. *Ensifer* was detected in the nodules of only two *A. cyclops* populations and one *A. saligna* population. All of these were harvested from plants grown in introduced range soils. Both *Bradyrhizobium* and *Ensifer* co-occurred only in one introduced *A. cyclops* population. Notably, *Aminobacter*, which is not listed as a known rhizobia genus (Weir, 2016), was the most common bacterial genus found in nodules, especially in *A. cyclops* introduced range populations. Although *Aminobacter* is not listed as rhizobial, there is at least one report from Europe (France) suggesting that some species from this genus may contain legume nodulating members (Maynaud *et al.*, 2012). Considering the prevalence of *Aminobacter* in legume soils and nodules based on our study, it is plausible that bacteria from this genus may be forming a symbiotic N-fixing relationship in Australia as well – this warrants further investigations.

Within *Bradyrhizobium*, analysis revealed that host species by location had a significant interactive effect on *Bradyrhizobium* composition. This was largely driven
by differences in nodule communities of *A. cyclops* from south-western Australia which varied considerably from the eastern native host species. Notably, NFB composition among the western natives’ (*A. cyclops, A. saligna* and *P. lophantha*) nodules in their native range did not differ significantly, suggesting that these three host species have similar *Bradyrhizobium* composition in their nodules in south-west Australia. This is highly likely as these species, especially *A. cyclops* and *A. saligna*, often co-occur on dunes.

Within host species, our results showed that *Bradyrhizobium* spp. composition was significantly different in introduced compared to native range sites for only one host, *A. longifolia*. This suggests that *A. longifolia* may be associating with new *Bradyrhizobium* species in its introduced range in south-west Australia. Our previous results (Birnbaum *et al.*, 2012) showed that rhizobial abundance was very similar across the ranges. However, we also found that *A. longifolia* grew significantly better in introduced range soils (Birnbaum *et al.*, 2012) suggesting that NFB may have been a contributing factor. Nevertheless, whether these *Bradyrhizobium* species in the introduced range are more effective at N-fixation, potentially facilitating the invasion success of *A. longifolia*, remains to be confirmed as we did not re-authenticate rhizobial strains to assess host-specificity or strain efficacy (Howieson *et al.*, 1995; Barrett *et al.*, 2015).

**Endophytes not known as rhizobia**

In this study, we found that nitrogen fixing (*nifH* gene present) endophytes not previously known to nodulate legumes comprised a large proportion of the OTU pool in both the soils and nodules of our five study species. These organisms appeared consistently in all nodules, and although they were identified consistently in soils,
they are not generally observed as the most abundant soil organisms when “universal”
(e.g., 16S rRNA) surveys are conducted. Their presence in nodules does not,
therefore, appear to be random. Although it is possible these organisms came to
dominate pots inoculated with legume soils and so are an artefact, this seems unlikely.
High diversity of bacterial endophytes, including those containing *nifH* genes, in
legume nodules has been previously reported also from Belgium (De Meyer et al.,
2015), China (Deng et al., 2011), Tunisia (Zakhia et al., 2006), Ethiopia (Aserse et
al., 2013) and Australia (Hoque et al., 2011). Although their diversity and importance
in nodules is less well described and understood compared to rhizobia, some authors
have suggested that non-rhizobial endophytes are an important component of root
nodules (Velázquez et al., 2013) and assist the host plant in stress tolerance (Andrews
et al., 2010), pathogen control (El-Tarabily et al., 2010) and growth promotion
(Ibáñez et al., 2009).
De Meyer and colleagues (2015) suggested that plants may select for specific
endophytes from the environment (e.g., rhizosphere) as they found strong clustering
of endophytic bacteria in native legumes’ nodules from Belgium. In this study, we
found some evidence for this in the Australian context, although only for one host
species, *A. cyclops*. For instance, in the nodules of *A. cyclops* grown in soil from its
introduced range, we found that *Ensifer* co-occurred with *Halomonas*,
*Amorphomonas, Azospirillum, Aminobacter, Rhodoblastus* and the most dominant
unclassified genus (except in soil for one site in the introduced range which was
dominated by *Aminobacter*).
However, in *A. cyclops* nodules collected from plants grown in native range soils, the
same grouping occurred, but without *Ensifer*. This suggests that *A. cyclops* is possibly
forming novel interactions with rhizobia in its introduced range. Remarkably, in some
instances bacteria not known to act as rhizobia were the predominant components of nodules. For example, nodules of *A. cyclops* and *A. saligna* from one native range population contained a large amount of *Rhodoblastus* sp. bacteria which for both host species co-occurred with an almost identical subset of other bacteria such as *Aminobacter, Xanthomonas* and two unclassified groups, suggesting plausible clustering in the nodules. Why we were unable to detect known rhizobial genera in these plants and why these bacteria, which are not known as rhizobia, are so dominant in these nodules remains to be studied. Notably, the plants from which the nodules were collected in an experiment described elsewhere (see Birnbaum *et al.*, 2012) appeared healthy and were likely receiving the nitrogen required for growth, suggesting these endophytes may be indirectly participating in N supply to host plant. Future culturing and trapping studies may further elucidate this. Overall though, our results highlight that culture based methods capture only a fraction of “true diversity” of bacterial communities in nodules.

We present a comprehensive analysis of NFB from nodules and soils of five invasive legumes in Australia. However, the results from this study should be interpreted with some caution for several reasons: 1) In this study, NFB refers to putatively nitrogen fixing bacterial communities. We acknowledge that there is a whole suite of genes responsible for biological nitrogen fixation (BNF) and presence of one gene (in this case *nifH*) provides partial evidence for BNF, but does not necessarily confirm it; 2) The detection of *nifH* gene did not confirm that BNF was actively occurring during sampling. It rather suggests the presence of organisms potentially involved in BNF; 3) The presence of diazotrophic non-nodulating bacterial lineages (e.g. *Xanthomonas*) in nodules suggests that these may simply be opportunistic bacteria that are thriving in the nutrient rich nodule environment rather than being true endosymbionts (Dudeja *et
al., 2012); 4) The use of nifH to infer bacterial identity is not perfect, especially for the taxonomy of Bradyrhizobium, because the nifH phylogenetic signal is not congruent with the 16S rDNA information on which the taxonomy is based (Haukka et al., 1998). Furthermore, gene transfer for nitrogen fixation and nodulation genes often occurs between bacteria, which further complicates identity inference from single genes (Laguerre et al., 2001). Lastly, our results are somewhat limited as we only had available information on bacterial communities from populations of each species and range (i.e. OTU pool) and not from individual trees because we binned samples per population. Having data from individual trees within population would have increased the resolution of our study and provided more in-depth analysis of N-fixing bacterial communities associated with each legume. Furthermore, our preference to transform data to presence/absence may have limited the interpretation power of our results. More comprehensive results could have been found using different sample methods that emphasize community comparisons with sequence abundances and not OTU pools and presence/absence data.

In conclusion, our results reveal that NFB communities across the Australian continent are homogenous and predominantly contain Bradyrhizobium as the main genus known to be rhizobial (i.e., nitrogen fixing and nodule forming) in both soils and nodules, as previously reported (Liesack & Stackebrandt, 1992; Lafay & Burdon, 2001; Stępkowski et al., 2012). Our findings also show that although these legumes encounter similar NFB in soils and nodules at the genus level, differences between ranges are apparent on a finer taxonomic scale for some host species, i.e. A. cyclops and A. longifolia. This suggests that how a host is characterised depends on the taxonomic level studied for microbial symbionts. Future research should identify whether there are specific NFB species that contribute disproportionately more to A.
longifolia growth and performance and thus its invasion success in Australia.

Furthermore, future research should use a variety of methodological techniques and data analysis tools to improve our understanding of invasive legume-rhizobia associations. Individual tree based data and reliable bacterial abundance data is needed to better understand the small scale bacterial diversity; we were able to provide a snapshot of the N-fixers diversity associated with legumes in Australia.

Importantly, we also describe a high number of putatively non-nodulating bacteria in the nodules. Given their consistent appearance, we suggest that they potentially play important, not yet understood, roles as plant symbionts and require further investigation. These endosymbionts contain nif genes, are likely to interact with known rhizobial genera and may directly and indirectly influence plant growth, nodulation, disease suppression and the colonization and invasion success of these legumes – this clearly warrants further investigations.

Acknowledgements

We thank Carla Harris and Paweł Waryszak for extensive help in the field and acknowledge Luke Barrett for helpful discussions on the statistical analysis. We are grateful to Niels Brouwers for help with the map. We also thank three anonymous referees for constructive comments that improved the manuscript. This work was funded by Macquarie University Research Excellence Scholarship to CB and by an Australian Research Council Discovery grant (DP0879494) to ML.

REFERENCES


Classification of rhizobia based on nodC and nifH gene analysis reveals a close phylogenetic relationship among Phaseolus vulgaris symbionts. Microbiology, 147, 981-993.


279 [Accessed on 20th of Jan, 2016].


282

283 Supporting Information

284 Additional Supporting Information may be found in the online version of this article:

285 Appendix S1 Details of hosts and obtained sequence reads for amplified nifH gene.

286 Appendix S2 PCA plots for soil and nodule NFB.

287

288 Biosketch

289 Christina Birnbaum is a post-doc at Murdoch University, Western Australia. She is broadly interested in plant-soil interactions and plant ecology. She began this study as part of her doctoral dissertation at Macquarie University, New South Wales, Australia.

290 Author contributions: All authors conceived the ideas; C.B. collected the data; C.B. and A.B. analysed the data; all authors contributed to writing the manuscript.
FIGURE LEGENDS

Figure 1. Map showing the 49 sites where seed and soil samples were collected for native and non-native populations of four *Acacia* species and *Paraserianthes lophantha* in south-east and south-west Australia.

Figure 2. Rarefaction accumulation curves along the number of sequences obtained from each species’ introduced and native range as detected in 49 soil samples. Upper and lower rows (L-R, in alphabetic order) show bacterial species accumulation curves for the three western Australian native host species – a) *Acacia cyclops*, b) *Acacia saligna* and c) *Paraserianthes lophantha* and for the two eastern Australian native host species – d) *Acacia longifolia* and e) *Acacia melanoxylon*, respectively. Grey and black lines indicate populations from native and introduced range, respectively. Curves are presented up to minimum of 474 sequences. For a total number of sequences see Appendix S1.

Figure 3. Heatmap with relative abundance data for nitrogen fixing bacterial communities detected in 49 soil samples. Host species names in bold indicate populations that nodules were collected from after a glasshouse experiment using field collected soil as inoculum. Asterisk on bacterial species names indicates groups that were also found in nodules. OTUs that had ≤ 1% abundance score were not included in the heatmap. *Acacia cyclops* (*A. cyc*), *Acacia saligna* (*A. sal*), *Paraserianthes lophantha* (*P. lop*), *Acacia longifolia* (*A. lon*) and *Acacia melanoxylon* (*A. mel*).
Figure 4. Rarefaction accumulation curves along the number of sequences obtained from each species’ introduced and native range as detected in 29 nodule samples. Upper and lower rows (L-R, in alphabetic order) show bacterial species accumulation curves for the three western Australian native host species – a) *Acacia cyclops*, b) *Acacia saligna* and c) *Paraserianthes lophantha* and for the two eastern Australian native host species – d) *Acacia longifolia* and e) *Acacia melanoxylon*, respectively. Grey and black lines indicate populations from native and introduced range, respectively. Error bars represent standard error. Curves are presented up to minimum of 1142 sequences. For a total number of sequences see Appendix S1.

Figure 5. Heatmap with relative abundance data for nitrogen fixing bacterial communities in Australia detected in 29 nodule samples. OTUs that had ≤ 1% abundance score were not included in the heatmap. N – native range, NN – non-native range.
Figure 2.
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<td>N</td>
</tr>
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<td>N</td>
</tr>
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<tr>
<td>91-100%</td>
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Genus (Family)

- Xanthomonas (Xanthomonadaceae)
- * unclassified (unclassified)
- Rhodoblastus (Bradyrhizobiaceae)
- * unclassified (unclassified)
- Aminobacter (Phyllobacteriaceae)
- * Pseudacidavorax (Comamonadaceae)
- * unclassified (Rubrivivax)
- Azospirillum (Rhodospirillaceae)
- * Bradyrhizobium (Bradyrhizobiaceae)
- * Amorphomonas (Rhizobiaceae)
- Halomonas (Halomonadaceae)
- Geo bacter (Geo bacteraceae)
- Pelobacter (Pelobacteraceae)
- * Mytilus (Opitutaceae)
- Allochromatium (Chromatiaceae)
- Azohydromonas (Alcaligenaceae)
- * Methylocystis (Methylocystaceae)
- Methanocaldococcus (Methanocaldococcaceae)
- * Alcaligenes (Alcaligenaceae)
- * Novosphingobium (Sphingomonadaceae)
- * Polaromonas (Comamonadaceae)
- * Ensifer (Rhizobiaceae)
- * Acenethiobacter (Xanthobacteraceae)
- * Agromyces (Microbacteriaceae)
- Rhodospirillum (Rhodospirillaceae)
- Desulfomonas (Desulfomonadaceae)
- Rhodopseudomonas (Bradyrhizobiaceae)
- * Magnetospirillum (Rhodospirillaceae)
- * Siphonbacillus (Siphonbacillaceae)
- Candidatus Desulfosporos (Peptococcaceae)
- * Alcaligenes (Alcaligenaceae)
- * Geobacter (Geo bacteraceae)
- * Delta (Comamonadaceae)
- * Xanthobacter (Xanthobacteraceae)
- * unclassified (Bacilliaceae)
- * Spirillum (Sphingomonadaceae)
- * Alkalibacter (Ectothiorhodospiraceae)
- * Methylocella (Beijerinckiaceae)
- * Phaeospirillum (Rhodospirillaceae)
- * Desulfobacterium (Desulfobacteriaceae)
- * Methanopyrus (Methanopyraceae)
- * Sulfitobacter (Rhodobacteriaceae)
- * Desulfobulbus (Desulfovibrionaceae)
- * unclassified (unclassified)
- * unclassified (Idonella)
- Marichromatium (Chromatiaceae)
- * Enelina (Enterobacteriaceae)
- * unclassified (unclassified)
- * Phyllobacterium (Phyllobacteriaceae)
Figure 4.
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Genus (Family)
- Aminobacter (Phyllobacteriaceae)
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- unclassified (Leptothrix)
- Xanthomonas (Xanthomonadaeae)
- Bradyrhizobium (Bradyrhizobiaceae)
- Pseudacidovorax (Comamonadaeae)
- Rhodoblastus (Bradyrhizobiaceae)
- Amorphone (Rhizobiaceae)
- Ensifer (Rhizobiaceae)
- unclassified (Rubrivivax)
- Novosphingobium (Sphingomonadaeae)
- Alcaligenes (Alcaligenaceae)
- Microbacteriaceae (Micrococcinaceae)
Nitrogen fixing bacterial communities in invasive legume nodules and associated soils are similar across introduced and native range populations in Australia

Christina Birnbaum, Andrew Bissett, Peter H. Thrall, Michelle R. Leishman

Appendix S1. Details of hosts and obtained sequence reads for amplified nifH gene from native and non-native soils (49) collected across south-east and south-west Australia and from nodules (29) harvested after a glasshouse experiment using field collected soil inoculum for four Acacia species and Paraseranthes lophantha. Number of OTUs (richness based on rarefaction) and Shannon diversity index are also presented for each species for native and introduced ranges (mean (±SE)).

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<th>Shannon diversity index</th>
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<td>4.</td>
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<td>9614</td>
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<td>WA</td>
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**A. saligna**

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<td>Diameter Mean ± SD (cm)</td>
<td>Biomass Mean ± SD (kg ha⁻¹)</td>
<td>Total Mean ± SD (kg ha⁻¹)</td>
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*A. melanoxylon*

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Appendix S2 PCA plots for soil and nodule nitrogen fixing bacterial communities based on nifH amplification from soils and nodules associated with four invasive Acacia species and Paraserianthes lophantha in Australia. Open and closed symbols represent native and non-native populations, respectively.

Figure S1. PCA plots for soil nitrogen fixing bacterial communities based on extracted DNA from soils associated with four Acacia species and Paraserianthes lophantha. Open and closed symbols represent native and non-native populations, respectively.

Figure S2. PCA plots for nodule nitrogen fixing bacterial communities based on extracted DNA from soils associated with four Acacia species and Paraserianthes lophantha. Open and closed symbols represent native and non-native populations, respectively.
Figure S1.
Figure S2.