1 Original Article

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

4 Running head: Bacterial communities of legumes in Australia

5

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

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28 **ABSTRACT**

29 **Aim**

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

38

39 **Location**

40 Australia.

41

42 **Methods**

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

48

49 **Results**

50 We found no difference in the NFB community of soils or nodules between native and
51 introduced ranges across the five species, suggesting that these legumes encounter
52 similar NFB communities in soils across Australia. *Bradyrhizobium* was the most
53 abundant rhizobial genus present in both soils and nodules. *Bradyrhizobium* species
54 found in nodules were significantly different across the ranges for *A. longifolia.*
55
56 **Main conclusions**
57 The results indicate that these invasive legumes have similar nitrogen fixing bacterial
58 communities in their rhizosphere and nodules across Australia, with the exception of
59 *A. longifolia*. This species has diverse *Bradyrhizobium* genotypes in its nodules
60 suggesting that *A. longifolia* may be a more generalist host compared to the other four
61 legumes. Thus it is unlikely that the invasive success of these legumes is constrained
62 by the absence of suitable bacterial symbionts in soil. Better knowledge of legume-
63 soil interactions could facilitate more informed and effective management of invasive
64 legumes in their introduced ranges in Australia and elsewhere.
65
66

67 **Introduction**

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

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

82 Generally, it has been proposed that invaders can be either constrained by mutualists
83 in the absence of suitable partners (Parker, 2001) or benefit from newly acquired
84 symbionts in novel ranges (Marler *et al.*, 1999; Parker *et al.*, 2007). There is certainly
85 evidence that lack of compatible key soil mutualists serves as a constraint for
86 successful establishment in the novel range for some species (Díez, 2005; Nuñez *et*
87 *al.*, 2009; Dickie *et al.*, 2010). Legumes have been reported to rely extensively on
88 mutualisms (e.g., rhizobia, mycorrhizal fungi) to successfully colonize and establish
89 in novel areas (Sprent & Parsons, 2000; Parker, 2001). Absence or low densities of
90 compatible rhizobia have been shown to limit range expansion and fitness of some

91 legume species (Stanton-Geddes & Anderson, 2011), including invasive Australian
92 acacias in New Zealand (Wandrag *et al.*, 2013).
93 Broad symbiotic promiscuity and ability to nodulate at low rhizobial abundance have
94 been described as significant advantages for invading legumes (Parker, 2001; Perez-
95 Fernanndez & Lamont, 2003; Rodríguez-Echeverria *et al.*, 2011). Previous studies
96 have shown that some invasive woody legumes are able to readily nodulate (Lafay &
97 Burdon, 2006) and associate with novel bacterial communities in their exotic ranges
98 (Marsudi *et al.*, 1999; Amrani *et al.*, 2010; Callaway *et al.*, 2011; Ndlovu *et al.*,
99 2013).
100 Despite the evidence for promiscuity for some host species, there are also reports
101 showing that some invasive legumes in their invasive range have specificity towards
102 rhizobia from their native range (Chen *et al.*, 2005). Such results were reported for *A.*
103 *longifolia* in Portugal which was found to associate with rhizobial communities that
104 were very similar to those from *A. longifolia*'s native range in south-east Australia
105 (Rodríguez-Echeverria, 2010). Thus, there appears to be considerable variation
106 between legume hosts and their symbiotic associations across introduced and native
107 ranges.
108 There is evidence to suggest that invasive acacias (sensu Richardson *et al*. 2011)
109 could be more promiscuous, i.e., they are able to associate with more diverse rhizobia,
110 than non-invasive acacias (Klock *et al.*, 2015). However, there is also evidence to
111 suggest that some acacias (e.g., *A. cyclops, A. pycnantha*) are able to non-specifically
112 nodulate with both fast- and slow-growing rhizobia in their novel ranges (Marsudi *et*
113 *al.*, 1999; Mohamed *et al.*, 2000; Lafay & Burdon, 2006; Ndlovu *et al.*, 2013). Thus,
114 quantification of the role of mutualists, such as rhizobia, in determining invasion

115 success could enhance our understanding of species' invasion potential more
116 generally.
117 In addition to known rhizobia, legume nodules harbour many other endophytes
118 (Hoque *et al.*, 2011; De Meyer *et al.*, 2015), whose role within plants is largely
119 unknown. Some authors have suggested that endophytes constitute an important
120 component of the nodule bacterial community (Velázquez *et al.*, 2013) and perform
121 important functions such as assisting the host plant in pathogen control (El-Tarabily *et*
122 *al.*, 2010) and growth promotion (Ibáñez *et al.*, 2009). To better predict the role of
123 bacterial communities in plant invasions, we need to understand the diversity and
124 function of nodule endophytes.
125 The primary aim of this study was to determine whether invasive Australian legumes
126 [i.e., *A. cyclops, A. longifolia, A. melanoxylon*, *A. saligna* and *Paraserianthes*
127 *lophantha* (hereafter collectively termed legumes)] encounter and associate with
128 different nitrogen-fixing bacterial (NFB) communities in soils and nodules in their
129 introduced range compared to native Australian range. We hypothesized that, because
130 these host species are widely distributed and considered invasive, they are likely to
131 accumulate different NFB nodule communities across introduced and native range
132 populations.
133

134 **Materials and methods**

135 **Study species**

136 Four *Acacia* species (*A. cyclops* A.Cunn. ex G.Don, *A. saligna* (Labill.) H.L. Wendl,
137 *A. longifolia* (Andrews) Willd. and *A. melanoxylon* R.Br.), and a close relative, *P.*
138 *lophantha* (Willd.) I.C. Nielsen, were chosen as host species. *Acacia cyclops*, *A.*
139 *saligna* and *P. lophantha* are native to Western Australia, but have been introduced to

140 the eastern Australian states (New South Wales, Victoria and South Australia) where
141 they have naturalised and become invasive. *Acacia longifolia* and *A. melanoxylon* are
142 native to south-east Australia, but have been introduced to and become invasive in
143 Western Australia.
144
145 **Study sites and soil sampling**
146 To characterise the diversity of organisms putatively capable of N fixation and
147 associated with our study species, we collected soil samples in December 2009 within
148 the native and introduced ranges of each of the five species, across south-eastern and
149 south-western Australia (Fig. 1 and details in Supporting Information Appendix S1).
150 For each species, with the exception of *A. melanoxylon* for which we only had four
151 populations in its introduced range, we sampled soil under five trees from each of five
152 populations within each range [5 species x 2 ranges (native and introduced) x 5
153 populations]. Thus, we sampled five populations in the native and introduced ranges
154 (n=5) for most species except *A. melanoxylon* (n=5 to 4, respectively). A total of 1000
155 g of soil was collected beneath each tree, as close to roots as possible, at a depth of
156 10-15 cm and then bulked for each population. Soils were kept in a cooler in the field
157 before being stored at 4°C. Soils were subsampled for genetic analysis within a week.
158 Soils were sieved to 2 mm to remove leaves and other coarse material and to
159 homogenise samples. Soil subsamples were placed in sterilized falcon tubes and
160 stored in the freezer at -20°C until further analysis. All sampling and processing
161 equipment, including sieves, were sterilized with 90% ethanol between populations.
162
163 **Nodule collection**

164 To assess putative nitrogen fixer diversity in nodules we extracted DNA from
165 nodules, collected from plant roots as part of a previous glasshouse experiment
166 designed to evaluate the role of soil microbial communities (predominantly rhizobia)
167 in determining cross-continental invasion success of the same five woody legume
168 species used in this study (see Birnbaum *et al*. 2012 for details). Depending on seed
169 availability, we had for *A. cyclops* n=4 (native) and n=3 (introduced), for *A. longifolia*
170 n=3 and n=4, for *A. melanoxylon* n=3 and n=2, for *A. saligna* n=2 and n=3 and for *P.*
171 *lophantha* n=2 and n= 3 populations, respectively. The plants in that experiment were
172 grown in field collected soils and assessed for growth in both introduced and native
173 range soils. From each plant 2-5 nodules were collected and surface sterilized with
174 90% ethanol and distilled water before being stored at -20°C in a plastic jar filled with
175 silica beads and cotton wool (Somasegaran, 1994). Nodules from 10 replicate plants
176 from each of 29 population/range soil combinations were pooled for DNA extraction.
177 Nodules (0.05 g) were crushed in liquid nitrogen to create a homogenised sample for
178 DNA extraction.
179

180 **Molecular analysis**

181 DNA from 49 soil and 29 nodule samples was isolated using a PowerSoil and
182 PowerPlant DNA isolation kit, respectively, following the manufacturer’s protocol
183 (MO Bio Laboratories, Inc. Carlsbad, CA). *NifH* was amplified from soils and nodule
184 DNA using a nested PCR with the internal primer pair *nifH* 1 (5´-TGY GAYCCN
185 AAR GCN GA-3´) and *nifH* 2 (5´-ADN GCC ATC ATY TCN C-3´) (Zehr &
186 McReynolds, 1989) and the external primers *nifH* 3 (5´-ATR TTR TTN GCN GCR
187 TA-3´) and *nifH* 4 (5´-TTY TAY GGN AAR GGN GG-3´). DNA was amplified
188 using the HotStarTaq Plus Master Kit (Qiagen, Valencia, CA) for PCR under

189 following conditions: 94°C for 3 minutes followed by 30 cycles of 94°C for 30
190 seconds; 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at
191 72°C for 5 minutes (Dowd *et al.*, 2008). Second PCR step was performed for 454
192 amplicon sequencing under the same conditions and primer set described above.
193 Following second PCR, all amplicon products from different samples were mixed in
194 equal volumes, and purified using Agencourt Ampure beads (Agencourt Bioscience
195 Corporation, MA, USA) (Dowd *et al.*, 2008). DNA fragments' size and concentration
196 were measured by using DNA chips under a Bio-Rad Experion Automated
197 Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380
198 Fluorometer (Turner Biosystems, CA, USA) before sequencing commenced using
199 bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to determine the
200 bacterial communities present according to the protocol in Dowd *et al*. (2008). More
201 details for methods used in the molecular analysis for PCR amplifications and
202 sequencing that were performed by the Research and Testing Laboratory (Lubbock,
203 Texas, USA) can be found in protocols described in Smith *et al*. (2010) and Dowd *et*
204 *al.* (2008).
205
206 **Bioinformatic analyses**
207 Downstream sequence analyses were performed on *nifH* sequences from nodules and
208 soils using MOTHUR 1.22.0 (Schloss *et al.*, 2009) following the adapted sequence
209 quality-control pipeline analysis described in detail in Schloss *et al.* (2011), until
210 chimeric sequences were removed. Sequences were included in subsequent analyses
211 only if they carried the correct primer sequence and were ≥150 bp and ≤400 bp long.
212 Following the pipeline analysis, sequences were blasted with a BLAST e-value >
213 0.001 against an existing *nifH* database that has 16 989 *nifH* sequences (Gaby &

214 Buckley, 2011). Only the sequences that returned a blast hit of sequence identity ≥
215 90% were kept and used for further downstream analysis. Approximately 13% of
216 sequences did not match any existing sequence in the database and thus were removed
217 from further analysis. Remaining sequences were checked for chimeras and a further
218 2% were removed from the dataset. After checking for chimeric sequences nodule and
219 soil *nifH* sequences were split into separate groups (nodules and soils) and pre-
220 clustered at 1% within each group. Following pre-clustering, nodule and soil
221 sequences were classified using *nifH* consensus taxonomy (Gaby & Buckley, 2011)
222 with the consensus confidence threshold of 51%. Furthermore, OTUs (Operational
223 Taxonomic Unit) containing only a single sequence (i.e. singletons) were omitted
224 from the analysis as they are likely to result from pyrosequencing errors (Tedersoo *et*
225 *al.*, 2010). The resultant OTU x sample matrix was binary transformed to
226 presence/absence and used for subsequent analyses to identify differences in NFB at
227 genus level. Additionally, to further explore the diversity of the dominant genus
228 known to symbiotically fix N_2 and nodulate with legumes (*Bradyrhizobium)*
229 sequences belonging to this genus were clustered at 97% sequence identity and
230 resultant OTU's analysed separately.
231
232 **Statistical analyses**
233 Principal coordinates analysis (PCA) based on a Bray-Curtis dissimilarity matrix was
234 carried out on presence/absence transformed data generated from the OTU species
235 matrix for soil and nodule NFB data separately. This allowed visual inspection to
236 identify differences between introduced and native range populations for all host
237 species. We used binary data to circumvent potential problems associated with

238 inferring abundances from amplion data and those associated with using proportional
239 data (relative abundance) (Amend *et al.*, 2010).
240 Upon inspection of PCA results, permutational multivariate analysis of variance
241 (Permanova) (Anderson, 2001) was used (9999 permutations) to test for differences in
242 microbial community composition among species, range (native versus introduced)
243 and location (south-east versus south-west) as well as their interactions. This analysis
244 was done separately for soil and nodule matrices.
245 Mantel tests (Mantel, 1967; Mantel & Valand, 1970) were performed to compare soil
246 and nodule dissimilarity matrices in order to determine if there was a correlation
247 between soil and nodule community composition. Additionally, the relative
248 abundance of NFB OTUs in soil and nodule data was calculated for each host species
249 and population. To estimate richness across host ranges, we used rarefaction analysis
250 to produce taxon accumulation curves using 1000 randomizations without
251 replacement. To estimate diversity among ranges, we calculated Shannon diversity
252 index. This metric allowed us to measure the OTU richness in a population of plants,
253 and compare the overall richness in populations between native and introduced ranges
254 per species.
255 PCA analysis and ordination, Mantel test and Shannon diversity were performed in
256 the R 3.1.1 programming language (R Core Team, 2015) using ‘vegan’ (Oksanen *et*
257 *al.*, 2011) and ‘BiodiversityR’ (Kindt & Coe, 2005) packages. Permanova analysis
258 was performed in PRIMER 6. Rarefaction analysis was conducted in MOTHUR 1.22.0.
259

260 **Results**

261 **Nitrogen fixing bacterial communities in soils**

262 A total of 119 NFB genera (from 358,349 sequences) were identified from soil
263 collected from the rhizospheres of the studied species, across both native and
264 introduced ranges. Of these, 24 genera (13.5% or 48,543 sequences) had no known
265 bacterial classification. Seven genera (13.3% or 47,779 sequences) are known to
266 nodulate and form symbiotic relationships with legumes (Weir, 2016). Of these seven
267 NFB genera, an overwhelming number of sequences (i.e. 94% or 45,095) belonged to
268 *Bradyrhizobium*. The majority of detected taxa in soil, i.e. 112 NFB genera (including
269 the unclassified sequences), belonged to genera not known to be rhizobial symbionts.
270 The overall Permanova model for soil NFB community composition at genus level
271 revealed a significant interaction between native range soils and host species,
272 suggesting that soil NFB composition is driven by larger geographic-scale variation
273 between the NFB communities of south-eastern and south-western Australia (Table
274 1).
275 Rarefaction analysis showed soil NFB taxon accumulation curves reached asymptotes
276 in all cases, indicating that sampling effort captured the majority of NFB diversity in
277 these samples (Fig. 2). Overall bacterial OTU richness at genus level was not
278 significantly different in soils across either host species or ranges (Appendix S1).
279 Pairwise analyses between *A. cyclops* and the other four host species [i.e., species x
280 location (south-east versus south-west)] showed that soil NFB composition at the
281 genus level for *A. cyclops* differed significantly from those of *A. melanoxylon, A.*
282 *saligna* and *P. lophantha* (Table 2). Overall, no differences in soil NFB composition
283 were detected within host species across their introduced and native ranges (Appendix
284 S2 Figure S1), suggesting that soil NFB composition is similar across these
285 geographically disjunct areas for these species, at least at the genus level.

286 The four most common and abundant soil bacterial genera (see Fig. 3) identified from
287 *nifH* sequences belonged to organisms that are not known to be legume symbionts.
288 Among the most common soil taxa that are classified as rhizobia (i.e., nitrogen fixing
289 and nodulating bacteria) (Weir, 2016) found from *nifH* sequences (Fig. 3), there were
290 seven OTUs belonging to the following genera: *Aminobacter*, *Azorhizobium*,
291 *Bradyrhizobium*, *Burkholderia*, *Ensifer*, *Methylobacterium* and *Phyllobacterium*.
292 *Aminobacter* is not listed in the Weir (2012) database as rhizobia. However there is
293 one report showing this genus could contain legume-nodulating members, and it is
294 closely related to another known legume-nodulating genus *Mesorhizobium* (Maynaud
295 *et al.*, 2012).
296 Because *Bradyrhizobium* was the most abundant confirmed rhizobial genus, we
297 subsequently analysed whether there was variation within this genus across ranges,
298 locations and host species. Results revealed that host species and location indeed had
299 a significant effect on *Bradyrhizobium* composition in the soil (Table 1), which was
300 driven by pair-wise differences between *A. cyclops* and the other four host species
301 (Table 3a). Differences in *Bradyrhizobium* composition across introduced and native
302 ranges was close to significant (α=0.05) only for one host species (*A. longifolia*, $t_{1,8}$ =
303 1.41, $P = 0.082$) out of the five studied legumes.
304

305 **Nitrogen fixing bacterial communities in nodules**

306 At genus level, a total of 29 genera (261,438 sequences) were classified as NFB
307 originating from legume nodules. Of these, six genera (38.9% or 101,952 sequences)
308 had no known bacterial classification. Five genera (34.7% or 90,906 sequences) were
309 previously known to nodulate and form symbiotic relationships with legumes:
310 *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Ensifer* and *Phyllobacterium* (Weir,

311 2016). *Burkholderia* and *Methylobacterium* were found in the soils, but were not
312 detected in the nodules. Of these five NFB genera, an overwhelming number of
313 sequences (i.e., 31% or 82,165) belonged to *Aminobacter. Bradyrhizobium* was the
314 second most common known rhizobial genus with 7,234 sequences (2.7 %). Similar to
315 soils, the majority of detected taxa in nodules (i.e., 24 genera, including the
316 unclassified sequences) belonged to genera not known as rhizobia (65.2% or 170,532
317 sequences).

318 The overall Permanova model for nodule NFB community composition did not reveal
319 any variation in NFB composition across host species and ranges at the genus level
320 suggesting that, broadly speaking, these five legumes contained similar pools of NFB
321 taxa in their nodules across introduced and native ranges (Table 1, Appendix S2
322 Figure S2). Rarefaction analysis showed that nodule NFB taxon accumulation curves
323 reached asymptotes in all cases, indicating that sampling effort captured the majority
324 of NFB taxa in these samples (Fig. 4). Overall bacterial OTU richness was not
325 significantly different across host species or ranges at the genus level (Appendix S1).
326 The five most abundant genera in nodules were similar to the five most abundant NFB
327 in soils (Fig. 5). Furthermore, all the taxa found in nodules were also found in soils,
328 and we found a significant correlation between the two matrices (i.e., nodule and soil
329 matrices) using the Pearson's product-moment correlation with 999 permutations
330 (Mantel statistic $r = 0.2347, P = 0.026$).

331 Similarly to soils, *Bradyrhizobium* was the most abundant confirmed rhizobial genus
332 in the nodules. Permanova analysis of variation within *Bradyrhizobium* revealed that
333 species and location interaction had a significant effect on nodule *Bradyrhizobium*
334 spp. composition (Table 1), which was driven by pair-wise differences between *A.*
335 *cyclops* and the other four host species (Table 3b). Additionally, *Bradyrhizobium* spp.

336 composition across introduced and native ranges was significantly different for one
337 host species *A. longifolia* ($t_{1,5} = 2.12, P = 0.024$) and close to significance for *P.*
338 *lophantha* ($t_{1,3} = 2.09, P = 0.058$).
339

340 **Discussion**

341 **Nitrogen fixing bacterial communities in soils**

342 Overall, at genus level, we found that host species by range had a significant
343 interactive effect on soil NFB composition. Pairwise analyses between all hosts and
344 ranges (native versus introduced) revealed that this effect was driven solely by one
345 host, *A. cyclops*, whose soil NFB communities predominantly from the introduced
346 ranges (i.e., Yorke Peninsula in South Australia) were significantly different from
347 those of *A. melanoxylon* (native range, south-east), *A. saligna* (native range, south-
348 west) and *P. lophantha* (native range, south-west). Notably, in our previous study, we
349 found that *A. cyclops'* soil fungal communities were also different from those of the
350 other four host species as well as between its own introduced and native range
351 populations (Birnbaum *et al.*, 2014). Thus it is plausible that *A. cyclops* has inherently
352 different soil microbial communities in its rhizosphere compared to the other four host
353 species.
354 We are not aware of other studies describing soil fungal or NFB communities of *A.*
355 *cyclops*. Limited published evidence on *A. cyclops* and its rhizobial communities from
356 nodules suggests that this species is non-specifically nodulated by both fast- and slow
357 growing rhizobia in its introduced range in Libya (Mohamed *et al.*, 2000). Estimates
358 of rhizobial abundance from introduced and native range soils in Australia have also
359 shown no differences between ranges (Birnbaum *et al.*, 2012) suggesting that *A.*

360 *cyclops* is not constrained by the absence of rhizobia and nodulates with diverse
361 rhizobia which may have contributed to its invasion success.
362 In terms of known nitrogen fixing and nodulating bacteria, we found three genera
363 (e.g. *Bradyrhizobium*, *Ensifer* (formerly *Sinorhizobium)* and *Azorhizobium*) to be
364 among the abundant groups in our soil samples. *Bradyrhizobium* has been frequently
365 reported to be one of the most common rhizobial genera in the nodules of *Acacia* spp.
366 in Australia (Stępkowski *et al.*, 2012), particularly in the south-eastern (Burdon *et al.*,
367 1999; Lafay & Burdon, 2001) and south-western (Marsudi *et al.*, 1999) regions, as
368 well as in introduced ranges in Portugal (Crisostomo *et al.*, 2013) and South Africa
369 (Ndlovu *et al.*, 2013). Thus, it is not surprising that it was found in the soils of all
370 species across introduced and native populations. *Ensifer*, on the other hand, was
371 found only in two western natives' (i.e., *A. cyclops* and *P. lophantha*) introduced
372 populations in the south-east, and was absent from native and introduced population
373 soils of *A. longifolia* and *A. melanoxylon*. Both *Bradyrhizobium* and *Ensifer* co-
374 occurred only in one introduced *A. cyclops* population, both in soils and nodules.
375 Although less common than *Bradyrhizobium*, *Ensifer* has been previously found in
376 the nodules of some *Acacia* spp. in south-eastern Australia (Hoque *et al.*, 2011) as
377 well as in introduced ranges in several African countries (Räsänen *et al.*, 2001;
378 Amrani *et al.*, 2010). *Azorhizobium* was the least common known rhizobial taxa found
379 in the soils of these legumes, and it has been linked more often to other leguminous
380 host species than acacias (Dreyfus *et al.*, 1988; Boivin *et al.*, 1997).
381 In general, we found no statistically significant differences in soil NFB communities
382 across introduced and native ranges within the five target plant species, suggesting
383 that these legumes encounter similar NFB communities in soils across the Australian
384 continent. Thus, it is unlikely that the absence of compatible bacterial communities

385 has constrained these species' naturalisation and invasion success in novel
386 environments across Australia. This is further evidenced by our previous findings that
387 most of these invasive legumes perform (based on biomass data) equally well in both
388 introduced and native range soils (Birnbaum & Leishman, 2013).
389

390 **Nitrogen fixing bacterial communities in nodules**

391 Overall, across our studied host species, we found no differences in NFB communities
392 at the genus level in nodules or between native and introduced ranges. In accordance
393 with the results from our analyses of associated soil communities, *Bradyrhizobium*
394 was the most common known rhizobial genus occurring in all species' introduced and
395 native range nodules, although considerably less *Bradyrhizobium* was detected from
396 *A. cyclops* nodules across both ranges. *Ensifer* was detected in the nodules of only two
397 *A. cyclops* populations and one *A. saligna* population. All of these were harvested
398 from plants grown in introduced range soils. Both *Bradyrhizobium* and *Ensifer* co-
399 occurred only in one introduced *A. cyclops* population. Notably, *Aminobacter*, which
400 is not listed as a known rhizobia genus (Weir, 2016), was the most common bacterial
401 genus found in nodules, especially in *A. cyclops* introduced range populations.
402 Although *Aminobacter* is not listed as rhizobial, there is at least one report from
403 Europe (France) suggesting that some species from this genus may contain legume
404 nodulating members (Maynaud *et al.*, 2012). Considering the prevalence of
405 *Aminobacter* in legume soils and nodules based on our study, it is plausible that
406 bacteria from this genus may be forming a symbiotic N-fixing relationship in
407 Australia as well – this warrants further investigations.
408 Within *Bradyrhizobium*, analysis revealed that host species by location had a
409 significant interactive effect on *Bradyrhizobium* composition. This was largely driven

410 by differences in nodule communities of *A. cyclops* from south-western Australia
411 which varied considerably from the eastern native host species. Notably, NFB
412 composition among the western natives' (*A. cyclops, A. saligna* and *P. lophantha*)
413 nodules in their native range did not differ significantly, suggesting that these three
414 host species have similar *Bradyrhizobium* composition in their nodules in south-west
415 Australia. This is highly likely as these species, especially *A. cyclops* and *A. saligna*,
416 often co-occur on dunes.
417 Within host species, our results showed that *Bradyrhizobium* spp. composition was
418 significantly different in introduced compared to native range sites for only one host,
419 *A. longifolia*. This suggests that *A. longifolia* may be associating with new
420 *Bradyrhizobium* species in its introduced range in south-west Australia. Our previous
421 results (Birnbaum *et al.*, 2012) showed that rhizobial abundance was very similar
422 across the ranges. However, we also found that *A. longifolia* grew significantly better
423 in introduced range soils (Birnbaum *et al.*, 2012) suggesting that NFB may have been
424 a contributing factor. Nevertheless, whether these *Bradyrhizobium* species in the
425 introduced range are more effective at N-fixation, potentially facilitating the invasion
426 success of *A. longifolia*, remains to be confirmed as we did not re-authenticate
427 rhizobial strains to assess host-specificity or strain efficacy (Howieson *et al.*, 1995;
428 Barrett *et al.*, 2015).
429

430 **Endophytes not known as rhizobia**

431 In this study, we found that nitrogen fixing (*nifH* gene present) endophytes not
432 previously known to nodulate legumes comprised a large proportion of the OTU pool
433 in both the soils and nodules of our five study species. These organisms appeared
434 consistently in all nodules, and although they were identified consistently in soils,

435 they are not generally observed as the most abundant soil organisms when "universal"
436 (e.g., 16S rRNA) surveys are conducted. Their presence in nodules does not,
437 therefore, appear to be random. Although it is possible these organisms came to
438 dominate pots inoculated with legume soils and so are an artefact, this seems unlikely.
439 High diversity of bacterial endophytes, including those containing *nifH* genes, in
440 legume nodules has been previously reported also from Belgium (De Meyer *et al.*,
441 2015), China (Deng *et al.*, 2011), Tunisia (Zakhia *et al.*, 2006), Ethiopia (Aserse *et*
442 *al.*, 2013) and Australia (Hoque *et al.*, 2011). Although their diversity and importance
443 in nodules is less well described and understood compared to rhizobia, some authors
444 have suggested that non-rhizobial endophytes are an important component of root
445 nodules (Velázquez *et al.*, 2013) and assist the host plant in stress tolerance (Andrews
446 *et al.*, 2010), pathogen control (El-Tarabily *et al.*, 2010) and growth promotion
447 (Ibáñez *et al.*, 2009).
448 De Meyer and colleagues (2015) suggested that plants may select for specific
449 endophytes from the environment (e.g., rhizosphere) as they found strong clustering
450 of endophytic bacteria in native legumes' nodules from Belgium. In this study, we
451 found some evidence for this in the Australian context, although only for one host
452 species, *A. cyclops*. For instance, in the nodules of *A. cyclops* grown in soil from its
453 introduced range, we found that *Ensifer* co-occurred with *Halomonas*,
454 *Amorphomonas*, *Azospirillum*, *Aminobacter*, *Rhodoblastus* and the most dominant
455 unclassified genus (except in soil for one site in the introduced range which was
456 dominated by *Aminobacter*).
457 However, in *A. cyclops* nodules collected from plants grown in native range soils, the
458 same grouping occurred, but without *Ensifer*. This suggests that *A. cyclops* is possibly
459 forming novel interactions with rhizobia in its introduced range. Remarkably, in some

460 instances bacteria not known to act as rhizobia were the predominant components of
461 nodules. For example, nodules of *A. cyclops* and *A. saligna* from one native range
462 population contained a large amount of *Rhodoblastus* sp. bacteria which for both host
463 species co-occurred with an almost identical subset of other bacteria such as
464 *Aminobacter*, *Xanthomonas* and two unclassified groups, suggesting plausible
465 clustering in the nodules. Why we were unable to detect known rhizobial genera in
466 these plants and why these bacteria, which are not known as rhizobia, are so dominant
467 in these nodules remains to be studied. Notably, the plants from which the nodules
468 were collected in an experiment described elsewhere (see Birnbaum *et al*., 2012)
469 appeared healthy and were likely receiving the nitrogen required for growth,
470 suggesting these endophytes may be indirectly participating in N supply to host plant.
471 Future culturing and trapping studies may further elucidate this. Overall though, our
472 results highlight that culture based methods capture only a fraction of "true diversity"
473 of bacterial communities in nodules.
474 We present a comprehensive analysis of NFB from nodules and soils of five invasive
475 legumes in Australia. However, the results from this study should be interpreted with
476 some caution for several reasons: 1) In this study, NFB refers to putatively nitrogen
477 fixing bacterial communities. We acknowledge that there is a whole suite of genes
478 responsible for biological nitrogen fixation (BNF) and presence of one gene (in this
479 case *nifH*) provides partial evidence for BNF, but does not necessarily confirm it; 2)
480 The detection of *nifH* gene did not confirm that BNF was actively occurring during
481 sampling. It rather suggests the presence of organisms potentially involved in BNF; 3)
482 The presence of diazotrophic non-nodulating bacterial lineages (e.g. *Xanthomonas*) in
483 nodules suggests that these may simply be opportunistic bacteria that are thriving in
484 the nutrient rich nodule environment rather than being true endosymbionts (Dudeja *et*

485 *al.*, 2012); 4) The use of *nifH* to infer bacterial identity is not perfect, especially for
486 the taxonomy of *Bradyrhizobium*, because the *nifH* phylogenetic signal is not
487 congruent with the 16S rDNA information on which the taxonomy is based (Haukka
488 *et al.*, 1998). Furthermore, gene transfer for nitrogen fixation and nodulation genes
489 often occurs between bacteria, which further complicates identity inference from
490 single genes (Laguerre *et al.*, 2001). Lastly, our results are somewhat limited as we
491 only had available information on bacterial communities from populations of each
492 species and range (i.e. OTU pool) and not from individual trees because we binned
493 samples per population. Having data from individual trees within population would
494 have increased the resolution of our study and provided more in-depth analysis of N-
495 fixing bacterial communities associated with each legume. Furthermore, our
496 preference to transform data to presence/absence may have limited the interpretation
497 power of our results. More comprehensive results could have been found using
498 different sample methods that emphasize community comparisons with sequence
499 abundances and not OTU pools and presence/absence data.
500 In conclusion, our results reveal that NFB communities across the Australian
501 continent are homogenous and predominantly contain *Bradyrhziobium* as the main
502 genus known to be rhizobial (i.e., nitrogen fixing and nodule forming) in both soils
503 and nodules, as previously reported (Liesack & Stackebrandt, 1992; Lafay & Burdon,
504 2001; Stępkowski *et al.*, 2012). Our findings also show that although these legumes
505 encounter similar NFB in soils and nodules at the genus level, differences between
506 ranges are apparent on a finer taxonomic scale for some host species, i.e. *A. cyclops*
507 and *A. longifolia*. This suggests that how a host is characterised depends on the
508 taxonomic level studied for microbial symbionts. Future research should identify
509 whether there are specific NFB species that contribute disproportionately more to *A.*

510 *longifolia* growth and performance and thus its invasion success in Australia.
511 Furthermore, future research should use a variety of methodological techniques and
512 data analysis tools to improve our understanding of invasive legume-rhizobia
513 associations. Individual tree based data and reliable bacterial abundance data is
514 needed to better understand the small scale bacterial diversity; we were able to
515 provide a snapshot of the N-fixers diversity associated with legumes in Australia.
516 Importantly, we also describe a high number of putatively non-nodulating bacteria in
517 the nodules. Given their consistent appearance, we suggest that they potentially play
518 important, not yet understood, roles as plant symbionts and require further
519 investigation. These endosymbionts contain *nif* genes, are likely to interact with
520 known rhizobial genera and may directly and indirectly influence plant growth,
521 nodulation, disease suppression and the colonization and invasion success of these
522 legumes – this clearly warrants further investigations.
523

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531

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757

758 **Supporting Information**

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

760

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

762 **Appendix S2** PCA plots for soil and nodule NFB.

763

764 **Biosketch**

765 **Christina Birnbaum is** a post-doc at Murdoch University, Western Australia. She is

766 broadly interested in plant-soil interactions and plant ecology. She began this

767 study as part of her doctoral dissertation at Macquarie University, New South

768 Wales, Australia.

769 Author contributions: All authors conceived the ideas; C.B. collected the data; C.B.

770 and A.B. analysed the data; all authors contributed to writing the manuscript.

771

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773

774 **FIGURE LEGENDS**

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

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

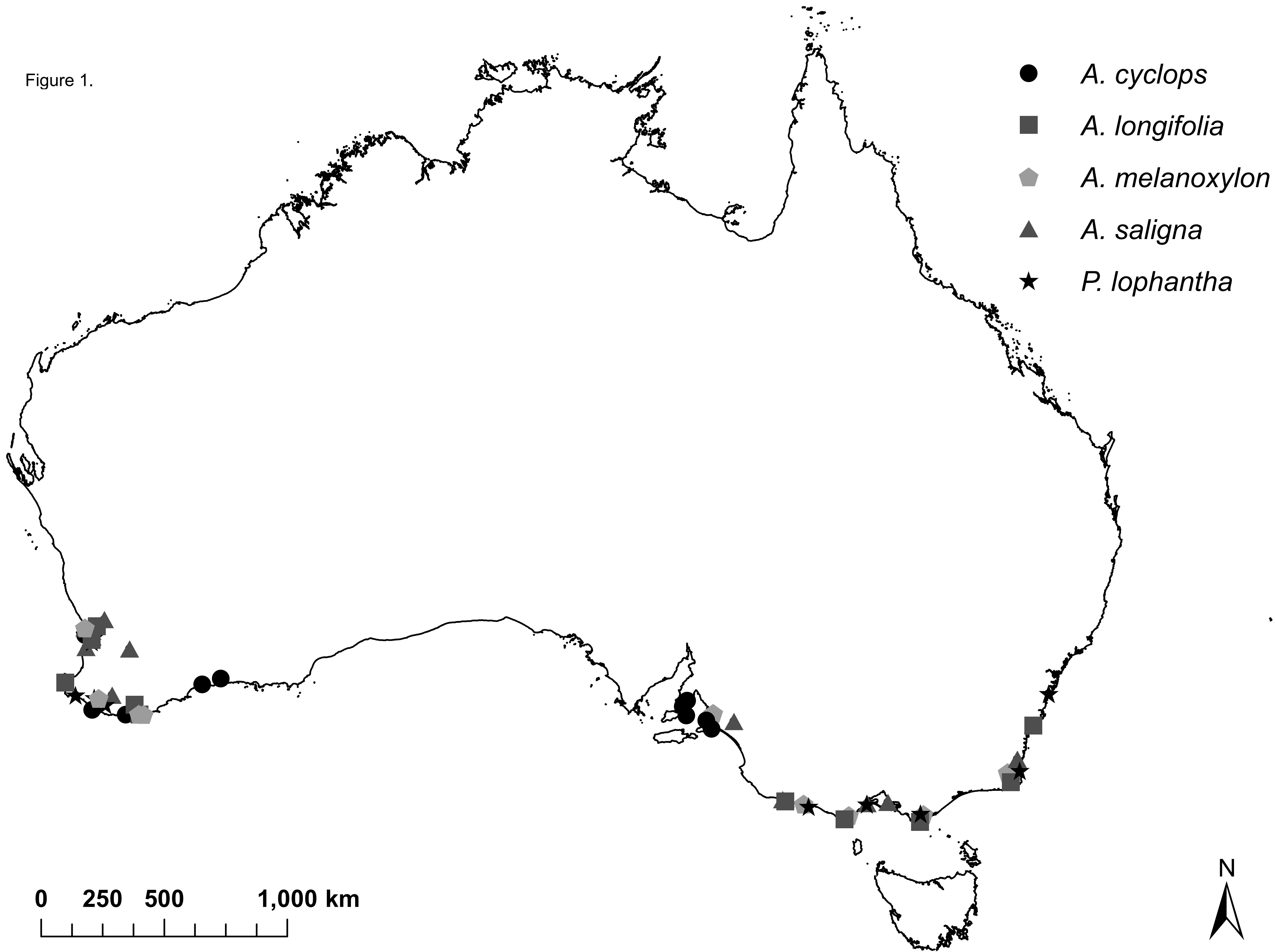
787

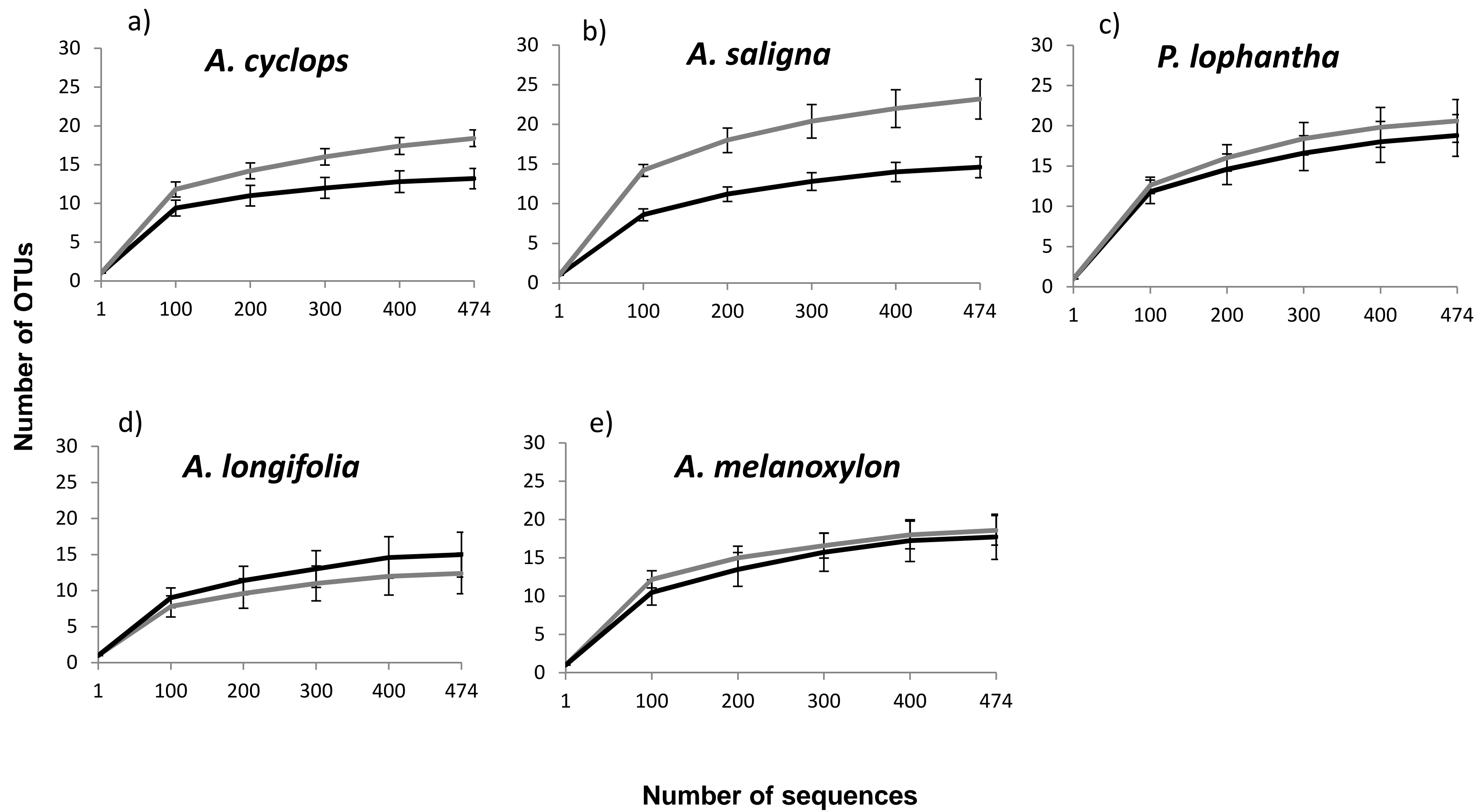
788 **Figure 3.** Heatmap with relative abundance data for nitrogen fixing bacterial
789 communities detected in 49 soil samples. Host species names in bold indicate
790 populations that nodules were collected from after a glasshouse experiment using field
791 collected soil as inoculum. Asterisk on bacterial species names indicates groups that
792 were also found in nodules. OTUs that had ≤ 1% abundance score were not included
793 in the heatmap. *Acacia cyclops* (*A. cyc*), *Acacia saligna* (*A. sal*), *Paraserianthes*
794 *lophantha* (*P. lop*), *Acacia longifolia* (*A. lon*) and *Acacia melanoxylon* (*A. mel*).

795

796 **Figure 4.** Rarefaction accumulation curves along the number of sequences obtained
797 from each species' introduced and native range as detected in 29 nodule samples.
798 Upper and lower rows (L-R, in alphabetic order) show bacterial species accumulation
799 curves for the three western Australian native host species – a) *Acacia cyclops*, b)
800 *Acacia saligna* and c) *Paraserianthes lophantha* and for the two eastern Australian
801 native host species – d) *Acacia longifolia* and e) *Acacia melanoxylon*, respectively.
802 Grey and black lines indicate populations from native and introduced range,
803 respectively. Error bars represent standard error. Curves are presented up to minimum
804 of 1142 sequences. For a total number of sequences see Appendix S1.
805
806 **Figure 5.** Heatmap with relative abundance data for nitrogen fixing bacterial
807 communities in Australia detected in 29 nodule samples. OTUs that had ≤ 1%
808 abundance score were not included in the heatmap. N – native range, NN – non-native
809 range.
810
811
812
813

Figure 1.





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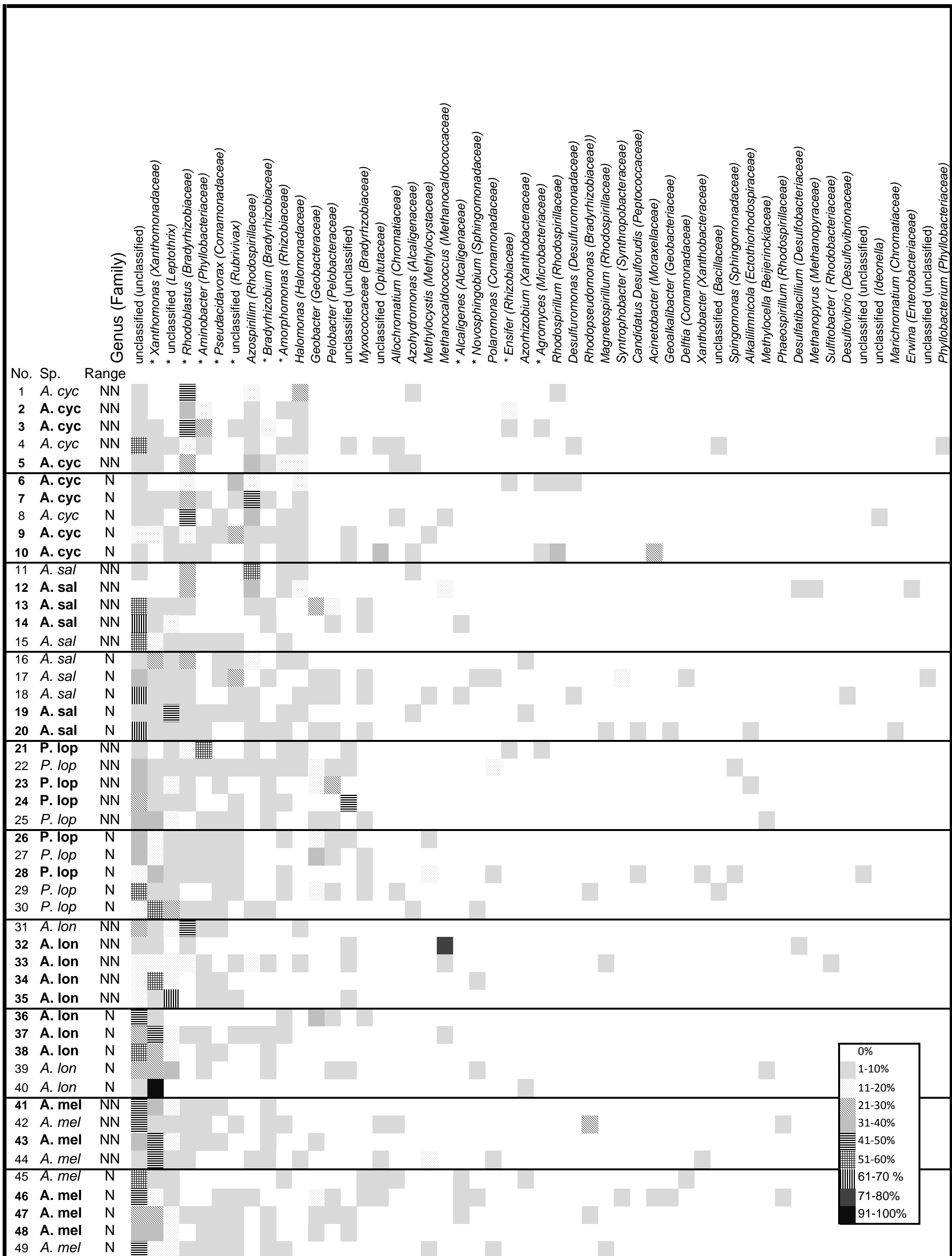

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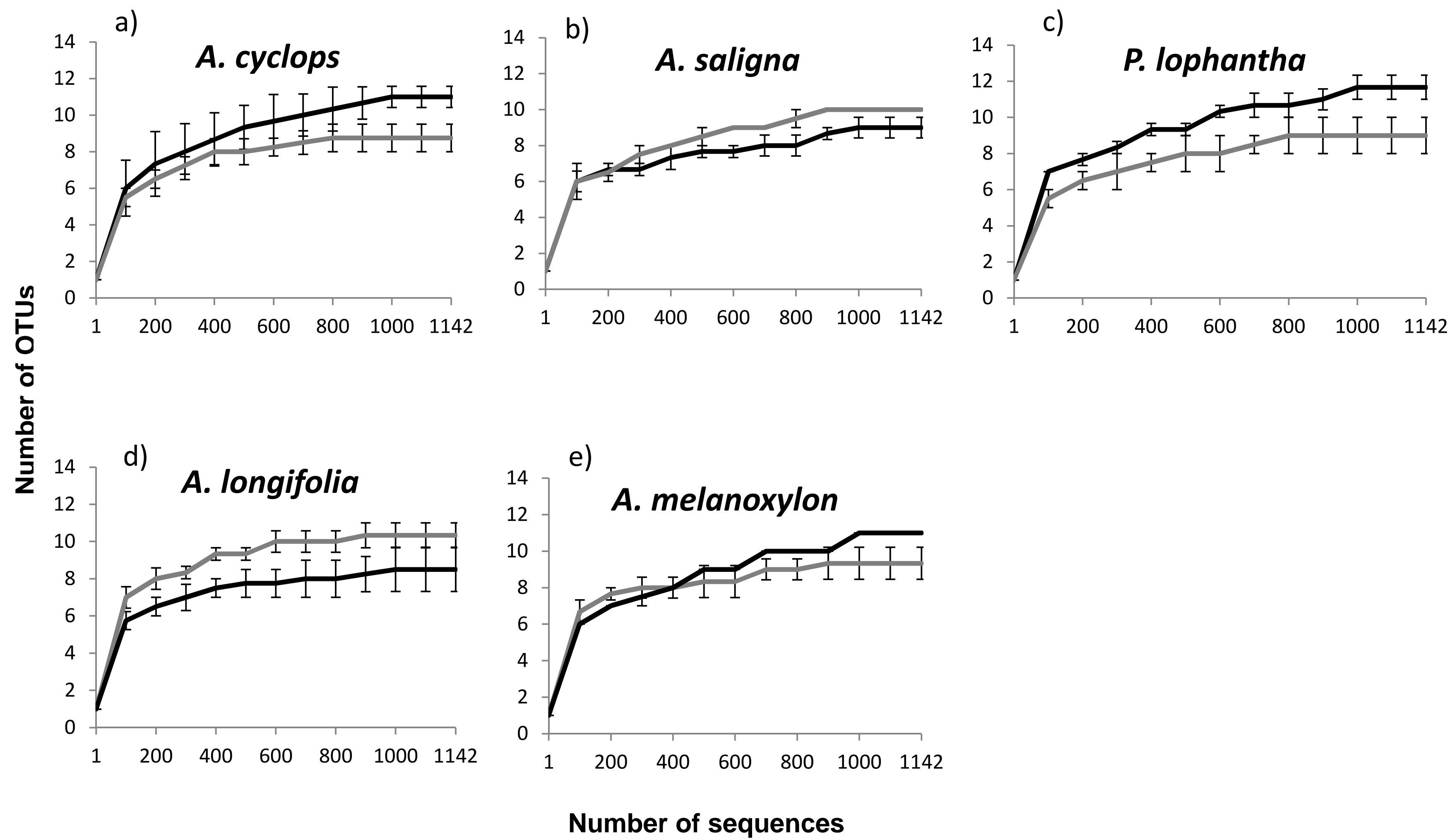

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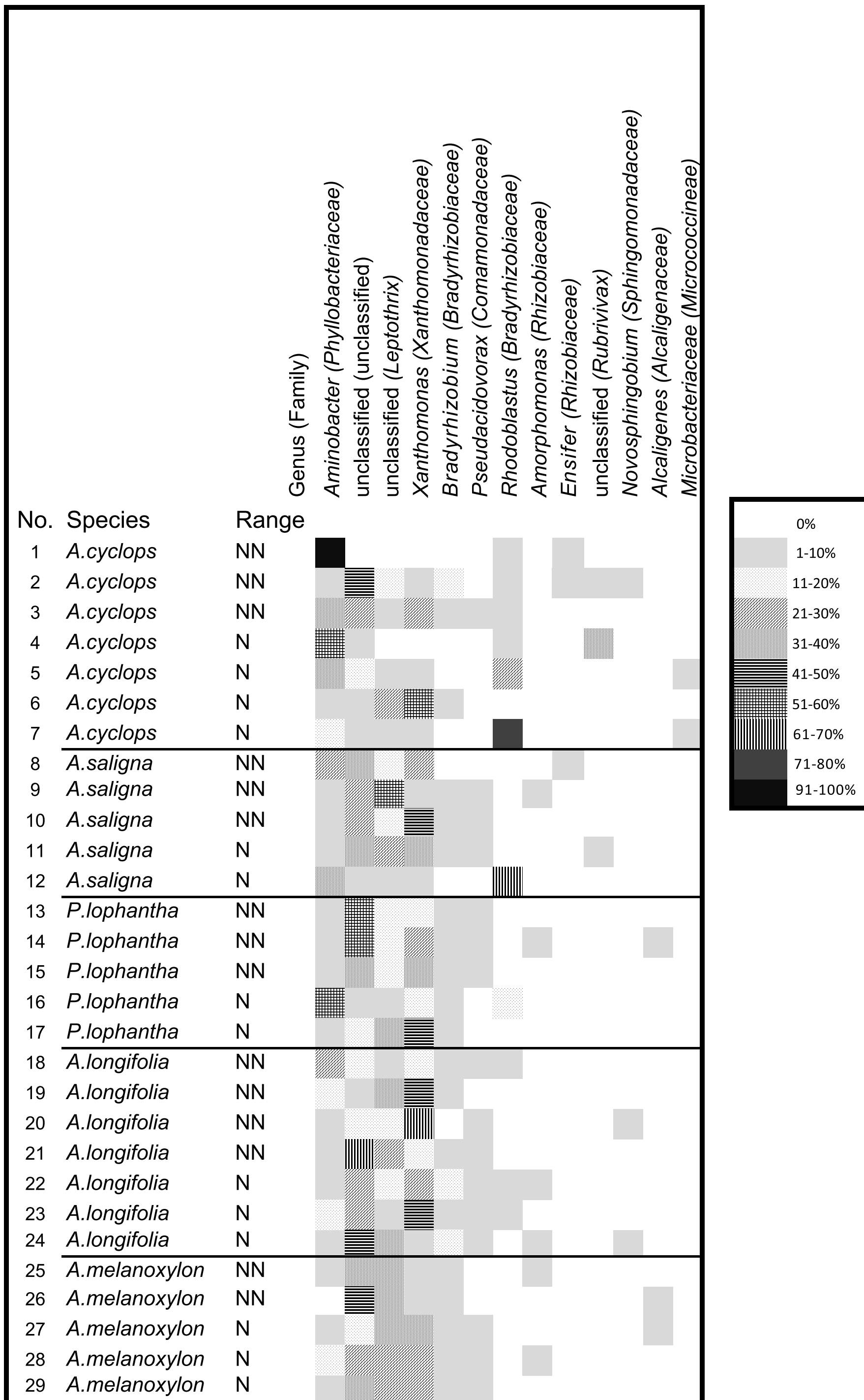

Figure 5.

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SUPPORTING INFORMATION

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)).

No.	Host plant species, populations	Location	Obtained sequence reads		State	Location	Number of OTUs		Shannon diversity index	
			Soils	Nodules			Soils	Nodules	Soils	Nodules
	A. cyclops									
1.	Native	South-west	23744	5993	WA	Ravensthorpe	18.4 (±1.07)	8.75 (±0.75)	3.57 (±0.25)	2.52 (±0.22)
2.		South-west	2815	3014	WA	Bremer Bay				
3.		South-west	13151	NA	WA	D`Entrecasteaux NP				

4.		South-west	9614	5415	WA	William Bay				
5.		South-west	8181	3427	WA	Coogee				
6.	Introduced	South-east	1847	NA	SA	Yorke peninsula	13.2 (±1.32)	11 (±0.57)	3.10 (±0.06)	3.12 (±0.11)
7.		South-east	3545	60108	SA	Yorke peninsula				
8.		South-east	2585	3940	SA	Yorke peninsula				
9.		South-east	1220	NA	SA	McLaren Vale				
10.		South-east	1714	35470	SA	Victor harbour				
A. saligna										
11.	Native	South-west	2102	NA	WA	Wickepin	23.2 (±2.51)	10 (±0)	4.02 (±0.17)	3.31 (±0.31)
12.		South-west	20858	NA	WA	Mordalup				
13.		South-west	13685	NA	WA	Yonderup				
14.		South-west	14975	23304	WA	Toodyay				
15.		South-west	12950	7590	WA	Perth				
16.	Introduced	South-east	5620	NA	NSW	Tailem Bend	14.6 (±1.32)	9 (±0.58)	3.52 (±0.42)	2.73 (±0.53)
17.		South-east	1751	1162	VIC	Portland-Nelson Rd				
18.		South-east	7643	24660	VIC	Surf coast hwy				
19.		South-east	5284	23890	SA	Mornington peninsula				

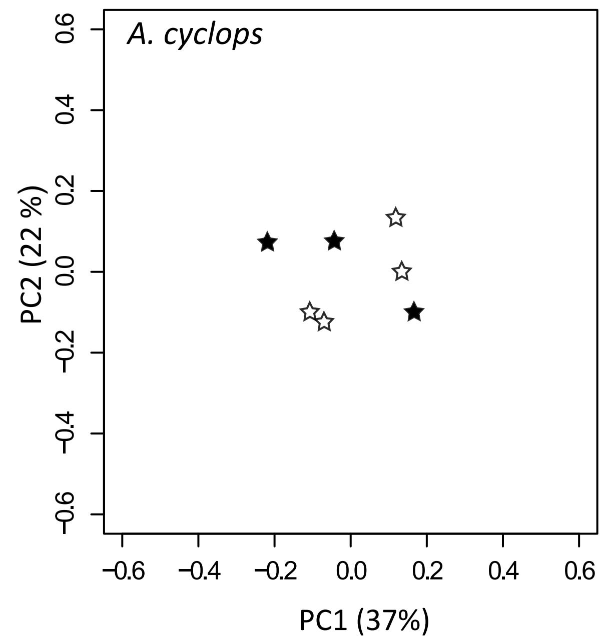
20.			South-east	3120	NA	SA	Bega				
	P. lophantha										
21.		Native	South-west	6664	3918	WA	Mt. Frankland NP	20.6 (±2.66)	9 (±1)	4.63 (±0.21)	3.01 (±0.07)
22.			South-west	6417	NA	WA	Pemberton				
23.			South-west	5654	4194	WA	Gingilup Swamps NR				
24.			South-west	17530	NA	WA	Serpentine NP				
25.			South-west	12694	NA	WA	Armadale				
26.		Introduced	South-east	3196	6361	VIC	Port Fairy	18.8 (±2.56)	11.7 (±0.67)	3.97 (±0.24)	3.22 (±0.17)
27.			South-east	2494	NA	VIC	Surf coast hwy				
28.			South-east	1477	2639	VIC	Toora				
29.			South-east	4311	2884	NSW	Eden				
30.			South-east	7825	NA	NSW	Scarborough				
	A. longifolia										
31.		Native	South-east	2250	4211	VIC	Portland-Nelson Rd	12.4 (±2.82)	10.3 (±0.67)	3.12 (±0.78)	3.14 (±0.25)
32.			South-east	42735	3078	VIC	Cape Otway				
33.			South-east	15169	2626	VIC	Wilsons Promontory				
34.			South-east	3258	NA	NSW	Croajingalong NP				

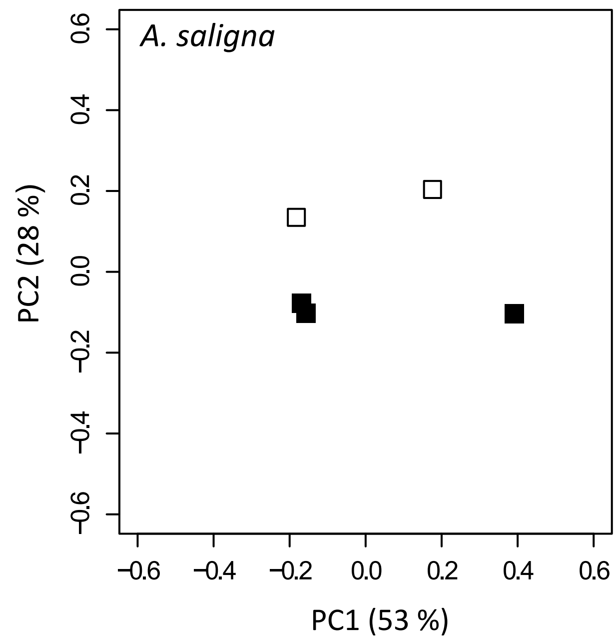
35.		South-east	5991	NA	NSW	Lake Tabourie				
36.	Introduced	South-west	5821	NA	WA	Lake Powell NR	15.0 (±3.11)	8.5 (±1.19)	3.52 (±0.37)	3.11 (±0.10)
37.		South-west	12113	4231	WA	Mt Barker				
38.		South-west	25388	6319	WA	Gracetown				
39.		South-west	16953	6161	WA	Watkins Road NR				
40.		South-west	18867	5466	WA	Gidgegannup				
	A. melanoxylon									
41.	Native	South-east	7644	NA	SA	Mt Lofty Summit	18.6 (±1.93)	9.3 (±0.88)	4.31 (±0.31)	3.56 (±0.14)
42.		South-east	73689	9153	VIC	Port Fairy				
43.		South-east	5330	8753	VIC	Apollo Bay				
44.		South-east	6351	6850	VIC	Toora				
45.		South-east	1754	NA	NSW	South East Forest NP				
46.	Introduced	South-west	6797	11780	WA	Albany	17.6 (±2.95)	11 (±0)	3.77 (±0.12)	2.71 (±0.41)
47.		South-west	2339	2305	WA	Elleker				
48.		South-west	3576	NA	WA	Quinninup				
49.		South-west	2755	NA	WA	Perth				

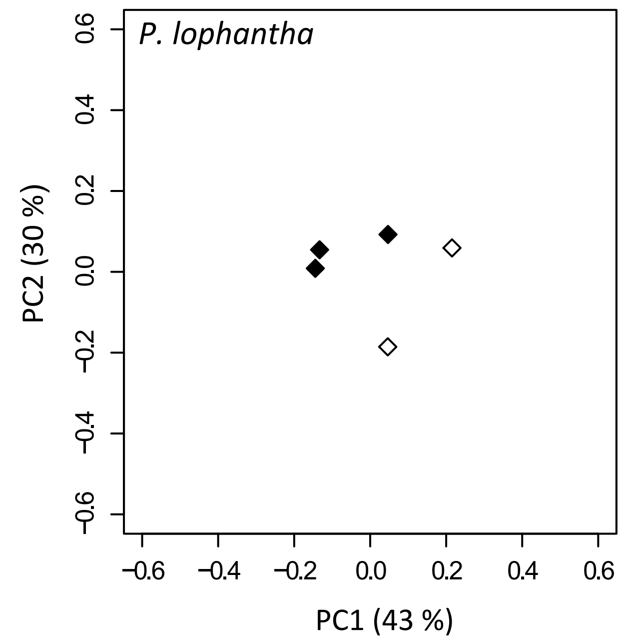
Appendix S2 PCA plots for soil and nodule nitrogen fixing bacterial communities based on *nifH* amplification from soils and nodules associated with four invasive *Acacia* species and *Paraserianthes lophantha* in Australia. Open and closed symbols represent native and non-native populations, respectively.

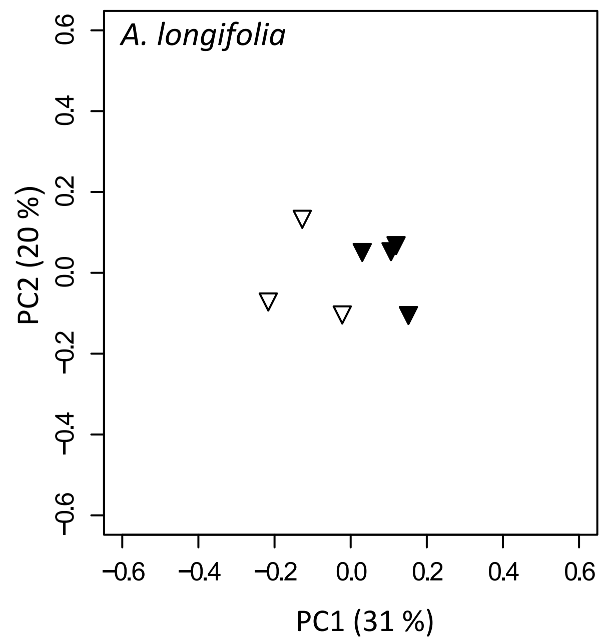
Figure S1. PCA plots for soil nitrogen fixing bacterial communities based on extracted DNA from soils associated with four *Acacia* species and *Paraserianthes lophantha*. Open and closed symbols represent native and non-native populations, respectively.

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.









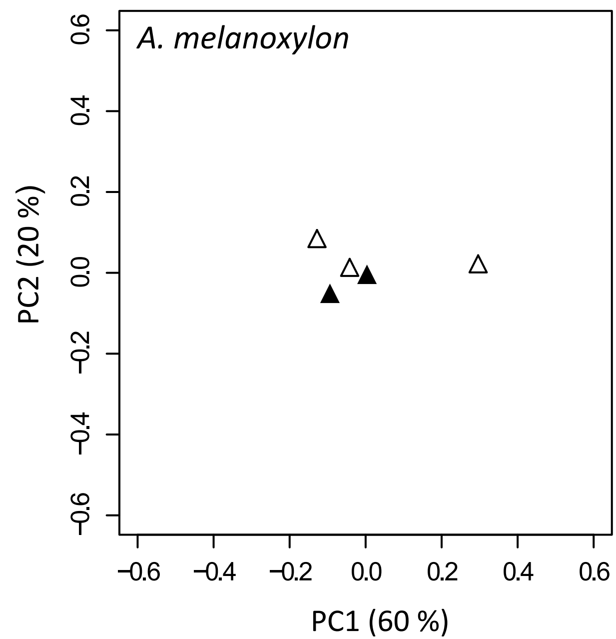
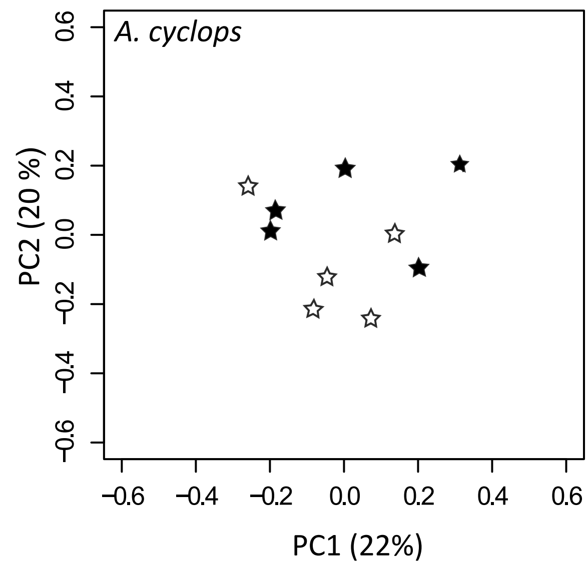
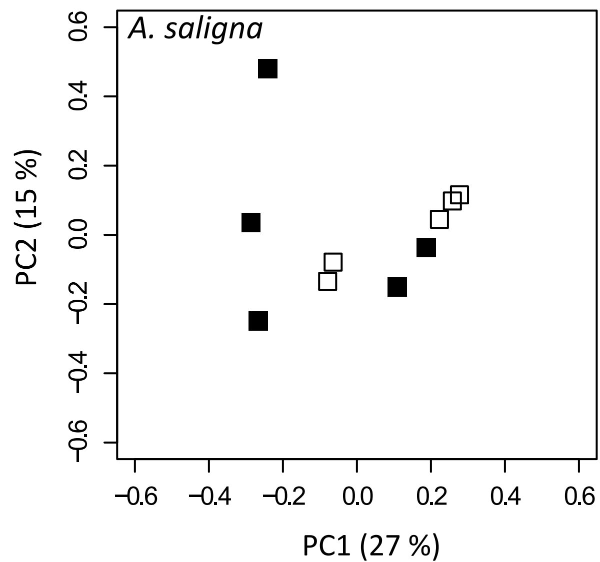
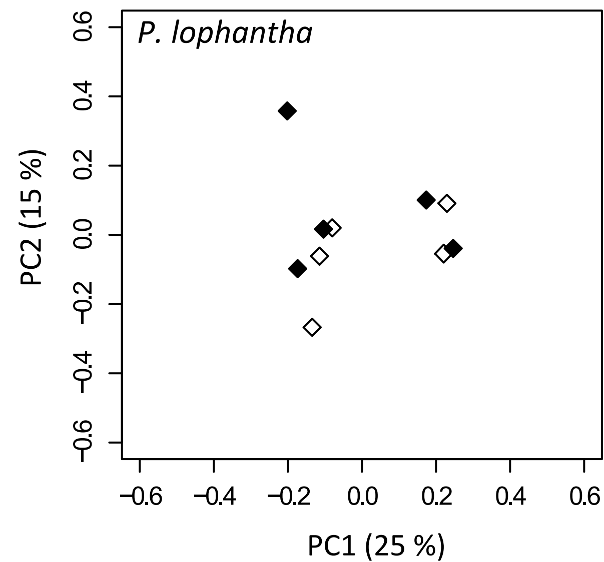
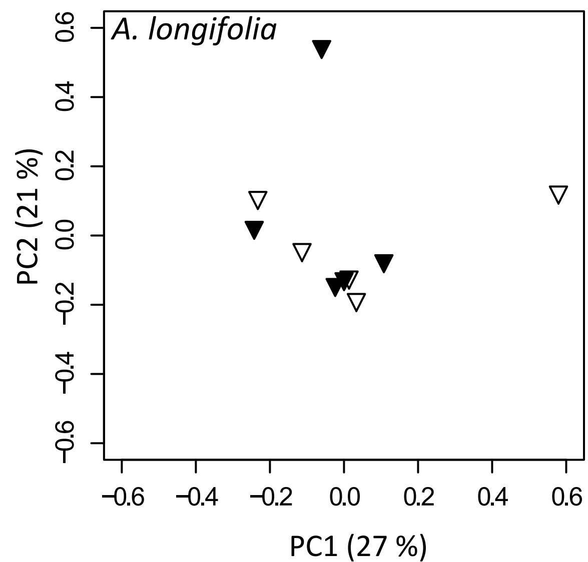


Figure S1.









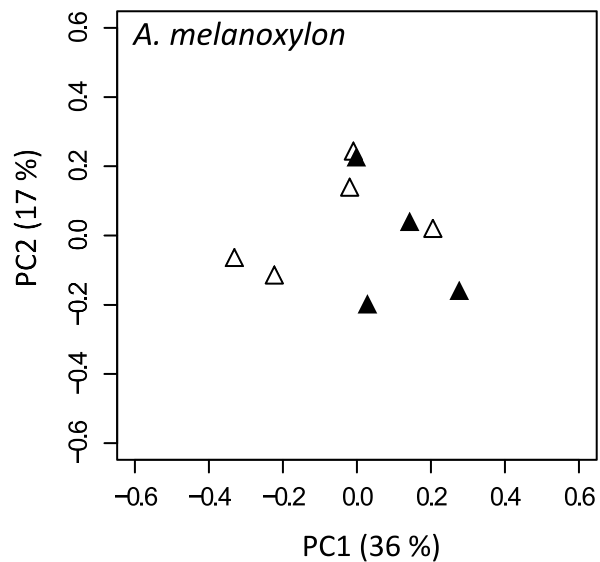


Figure S2.