Symbiotic N_2-fixer Community Composition, but Not Diversity, Shifts in Nodules of a Single Host Legume Across a 2-million-year Dune Chronosequence

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Abstract

Long-term soil age gradients are useful model systems to study how changes in nutrient limitation shape communities of plant root mutualists because they represent strong natural gradients of nutrient availability, particularly of nitrogen (N) and phosphorus (P). Here, we investigated changes in the dinitrogen (N_2)-fixing bacterial community composition and diversity in nodules of a single host legume (*Acacia rostellifera*) across the Jurien Bay chronosequence, a retrogressive 2 million-year old sequence of coastal dunes representing an exceptionally strong natural soil fertility gradient. We collected nodules from plants grown in soils from five chronosequence stages ranging from very young (10's of years; associated with strong N limitation for plant growth) to very old (>2,000,000 years; associated with strong P limitation), and sequenced the *nifH* gene in root nodules to determine the composition and diversity of N_2-fixing bacterial symbionts. A total of 335 unique *nifH* gene operational taxonomic units (OTUs) were identified. Community composition of N_2-fixing bacteria within nodules, but not diversity, changed with increasing soil age. These changes were attributed to pedogenesis-driven shifts in edaphic conditions, specifically pH, exchangeable manganese, resin-extractable phosphate, nitrate and nitrification rate. A large number of common N_2-fixing bacteria genera (e.g. *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*) belonging to the Rhizobiaceae family (α- proteobacteria) comprised 70% of all raw sequences and were present in all nodules. However, in the oldest soils, which show some of the lowest soil P availability ever recorded, harboured the largest proportion of unclassified OTUs, suggesting a unique set of N_2-fixing bacteria adapted to extreme P limitation. Our results show that N_2-fixing bacterial composition varies strongly during long-term ecosystem development, even within the same host, and therefore rhizobia show strong edaphic preferences.

Introduction

Plants form root symbioses with a number of soil micro-organisms that assist them in acquiring nitrogen (N) and phosphorus (P) [1]. One of these symbiotic associations forms between legumes and symbiotic N_2-fixing bacteria, collectively termed rhizobia. Rhizobia colonize roots to form root nodules, within which they use photosynthates to reduce atmospheric N_2 to ammonia, which is then used by plants to build amino acids and nucleotides [2].Rhizobia comprise a phylogenetically diverse group of α- and β-proteobacteria, with 13 genera and 98 species currently described, and many more still undescribed [3]. The majority of these bacterial species belong to the Rhizobiaceae family (α-proteobacteria) and are primarily associated with four common genera, i.e., *Rhizobium*, *Mesorhizobium*, *Ensifer*, and *Bradyrhizobium* [3].

Even though the symbiosis between plants and rhizobia is generally considered to be mutualistic, the amount of N_2 fixed by these associations can vary widely, and the symbiosis can sometimes even be parasitic [5,4,1]. For example, in black wattle (*Acacia mearnsii*) plantations, N_2 fixation can reach 200 kg N ha^{-1} [6]. Others have shown that endophytic N_2-fixers can contribute from 60 kg N ha^{-1} $year^{-1}$ in field studies [7] to 150 kg N ha^{-1} $year^{-1}$ in container studies [1]. Some studies have found that in high N environments the benefit of rhizobia to *Medicago truncatula* plant fitness was lower, suggesting that removing N changes the mutualism properties of rhizobia [8-10]. Rhizobia, specifically representatives from genus *Bradyrhizobium*, can be symbiotic and non-symbiotic soil inhabitants [11,12], although N_2 fixation is only known to occur within the symbiosis in the host. Host plant species, rhizobial strain identity and spatial heterogeneity in the soil abiotic environment all determine whether the legume-rhizobia symbiotic association is mutualistic or parasitic [13,8]. These shifts in the strength and direction of the interactions can be linked to shifts in bacterial composition

and activity [14,15], but environmental controls over shifts in rhizobial composition remain unclear. Nodule rhizobial diversity is mainly determined by edaphic conditions, nodule number and host / symbiont specificity [5,16,17]. Controlling for host identity can help determine the independent influence of edaphic conditions on rhizobial composition and diversity. While fertilisation experiments have quantified changes in rhizobial communities in experimentally manipulated and pasture soils [18,19], studies across natural soil fertility gradients are rare. As a result, we lack knowledge about edaphic drivers of N_2-fixing bacterial communities in natural environments.

Long-term gradients of soil age (i.e., soil chronosequences) are useful model systems to study edaphic drivers of plant-soil mutualisms and elucidate how nutrient limitation shapes these interactions over long periods [20,22,21,23]. Long-term soil chronosequences represent strong natural gradients in soil nutrient limitation [20,24] while minimising variation in other important abiotic conditions (e.g., parent material, climate, and topography; [25,20]). During long-term ecosystem development, major changes in soil nutrient availability occur [26,27,24] and can cause shifts in soil microbial communities [28,21,29,23]. For example, young soils in the Jurien Bay dune chronosequence in southwest Australia are relatively P-rich, but N-poor, while very old, i.e., > 2000 ka soils from this chronosequence are severely P- and N-impoverished (Table 1) [30].

Plant growth on young soils during primary succession tends to be N-limited, since N enters ecosystems primarily through biological N_2-fixation [32]. By contrast, plant productivity on older soils becomes increasingly P-limited, as P availability declines with soil age [33,30] whereas N input continues through biological nitrogen fixation. These changes in soil nutrient availability and limitation have been shown to strongly affect vegetation [34-37] and arbuscular mycorrhizal (AM) [21] and ectomycorrhizal (ECM) fungal communities [31]; however, effects on N_2-fixing rhizobial communities remain unknown. Albornoz *et al*. (2016)

reported a decline in nodulation of *Acacia rostellifera* Benth. (Fabaceae) with increasing soil age along the Jurien Bay dune chronosequence, suggesting that plant growth on the oldest soils is P, rather than N, limited, and that nodulation might be constrained by the high P demand for N_2 fixation [39,38,23]. Despite demonstrated changes in nodulation rates along this chronosequence, changes in rhizobial communities have not been explored.

In this study, we investigated changes in symbiotic community composition and diversity in *A. rostellifera* nodules harvested from a glasshouse experiment where plants were grown in field-collected soil from the Jurien Bay dune chronosequence [30,36,24]. We targeted the *nifH* gene from nodules because it is a nitrogen fixation marker, for which the largest number of rhizobial sequences is available for comparison [40,41] and to restrict sequencing to putative symbiotic N_2-fixers. Considering the ubiquity of native *Acacia* species in many Australian ecosystems with nutrient poor soils [42] and their widespread association with rhizobia, *Acacia*-rhizobia is an excellent model system to study the effects of edaphic factors on root symbioses [43], and to further elucidate how shifts in abiotic conditions affect nodule symbiotic community composition. In this study, we controlled for shifts in host composition by having a single host species (one of the few plant species occurring across most of the chronosequence; [37], thus allowing us to evaluate the effect of soils on rhizobial communities in nodules independently of host effects.

We predicted that large changes in soil chemistry along this dune chronosequence [30,24] would have a strong influence on nodule rhizobial composition. In particular, it is well documented that rhizobial diversity in soils correlates negatively with soil pH. For example, some rhizobia are unable to persist and fail to nodulate plants in soil of pH less than 5 [44,45]. There is a marked shift in soil pH in our soils from alkaline (youngest soils; pH 8.2) to acidic (oldest soils; pH 4.8) [30,24], therefore we hypothesized that rhizobial diversity in nodules will decrease along this gradient.

Methods

Study Area and Study Species

The study area is located in south-western Australia (~200 km north of Perth), along the Jurien Bay >2-million year dune chronosequence [30]. The coastal dune chronosequence is approximately 15 km wide, runs approximately parallel to the coast and comprises a series of three main dune systems of marine origin (i.e., Quindalup (young, medium and old), Spearwood and Bassendean) [46]. A detailed characterisation of the dune chronosequence is described elsewhere [30,24]. A summary of main characteristics of each soil chronosequence stage (i.e., stage 1 being the youngest, stage 5 the oldest), their estimated soil ages and most likely limiting nutrients is shown in Table 1. Briefly, soil age increases with distance from the coast and the soil age gradient corresponds to large changes in soil nutrients [30,24], whereby N is limiting in very young dunes (0.05 ka), intermediate-aged dunes (1 – 6.7 ka) are associated with co-limitation by N and P, and old dunes (125 – >2000 ka) are associated with strong P limitation (Table 1). Soil pH ranges from alkaline (8.2) to acidic (4.8) in youngest to oldest soils, respectively [24].

The climate in the area is Mediterranean, with a winter wet season (June-August) and extended seasonal summer drought [24]. There are no significant differences in precipitation across the chronosequence. The mean annual average temperature is 25°C (Australian Bureau of Meteorology, www.bom.gov.au/climate/data). Plant species diversity on these dunes has been found to increase with declining soil P availability [47,37]. The most abundant families by relative cover that occur across all chronosequence stages are the Myrtaceae, Fabaceae and Proteaceae [37]. Stages 4–5 are dominated by the Proteaceae (28%) and Fabaceae (24%), whereas Goodeniaceae has the highest relative cover (19%) in stage 1. Shrubs had the highest mean relative cover (55%), followed by sedges (13%), trees (12%) and perennial herbs (10%)

144 (for details see Fig.S7 in [35]). In the region, fire is the main disturbance to vegetation, with
145 fire-return intervals of <30 years [48,49].

146 To control for host plant identity, and hence focus on the effects of soil age on changes in
147 symbiotic rhizobial diversity and composition, we selected one of the very few plant species
148 that naturally occurs across most of the chronosequence stages, *A. rostellifera* [37].
149 Specifically, *A. rostellifera* has a relative cover of at least 1 % in soil stages 1 (14%), 2 (5%),
150 3 (6%) and 4 (6%) and 5 (~ <0.1%) (see Table S2 in Zemunik *et al*. 2016). *Acacia*
151 *rostellifera* belongs to the Fabaceae Lindl. and is native to Western Australia. In addition to
152 rhizobial associations, it forms AM and ECM associations [23]. It is a dense shrub or tree, 1
153 to 6 m high, and mainly grows on coastal dunes (FloraBase—the Western Australian Flora,
154 https://florabase.dpaw.wa.gov.au/).

155 Although the rhizobial diversity in the nodules of many Australian *Acacia* species has been
156 described [50,16,51], there are relatively few published reports describing specifically the
157 rhizobial diversity in *A. rostellifera* nodules, thus we cannot conclude whether *A. rostellifera*
158 is a specialist or generalist host. However, there is one report showing that *Ensifer sp*.,
159 *Rhizobium sp*., and *Bradyrhizobium sp*. nodulate with *A. rostellifera* [52], suggesting that it is
160 likely a generalist host and thus a suitable plant species to understand environmental controls
161 over rhizobial diversity.

162 **Soil collection**

163 Soils were collected in November 2013 from five soil chronosequence stages for the
164 glasshouse experiment (Table 1). From each chronosequence stage, we selected five plots (10
165 × 10 m) from the same set of permanent plots used in earlier studies [36,53,24,47,37]. In each
166 plot, we collected 5.3 l of bulk soil, from the top 30 cm layer from three points around the

plot. The three samples were then mixed and bulked for each plot. Soil was stored for ten days at 4°C before being used for the glasshouse experiment.

Glasshouse experiment

We targeted nodules of *A. rostellifera* to trap organisms that have N_2-fixing capacity and form a symbiotic association with the host plant, rather than sequencing rhizobia directly from the soils, thereby sampling only putative symbiotic N_2 fixers. *Acacia rostellifera* seeds were germinated in triple-steam pasteurised (80°C for two hours every two days over a period of six days) sand at 19°C with a 12 h photoperiod. Twenty days after germination, healthy seedlings were transplanted into experimental soil-filled 1 l pots and watered to 75% field capacity throughout the duration of the experiment, for a total of 25 pots (i.e., 5 dune stages × 5 replicates). After 21 weeks of growth in the glasshouse, seedlings were harvested and shoots separated from roots. Soil attached to roots was carefully washed away from root systems over a 1-mm sieve and all nodules removed. From each of the five replicate plants 2–3 nodules were collected, surface sterilised with 90% ethanol and distilled water before being stored in the freezer at -20°C in 1.5 ml Eppendorf tubes. Equipment and working surfaces were thoroughly cleaned with 70% (v/v) ethanol between each root system harvested, thus removing any tissue and DNA from the previous root system. One *Acacia rostellifera* replicate seedling died when grown in the oldest Bassendean soil, thus nodules were collected only from four replicates for the oldest soil treatment (n=24).

Molecular analysis

Thawed nodules were crushed with mortar and pestle to create a homogenised sample per replicate for DNA extraction. DNA was isolated from 0.03 g (±0.003 SE) fresh nodule tissue using the PowerSoil DNA isolation kit (MO Bio Laboratories, Inc. Carlsbad, CA), following

the manufacturer's protocol. The *nifH* gene was amplified from nodule DNA using the *nifH* 1F (5´-TGY GAYCCN AAR GCN GA-3´) and *nifH* 2R (5´-ADN GCC ATC ATY TCN C-3´) primer pair [54]. DNA was amplified using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 seconds and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple barcoded samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA). The pooled and purified PCR product was then used to prepare DNA libraries by following Illumina TruSeq DNA library preparation protocol (V2.1.3). DNA amplification and sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq following the manufacturer's guidelines.

Bioinformatic analyses

nifH paired end sequences were merged using FLASH [55]. Sequences containing ambiguous bases and homopolymer runs >10 bp were removed. Remaining sequences were clustered at 97% sequence identity using UPARSE [56] and an Operational Taxonomic Unit (OTU) table produced by mapping reads to the resultant OTUs using USEARCH [57]. Singleton OTUs were omitted from the analysis. Sequence data was subsampled without replacement (15259 sequences per sample) to ensure equal representation across all samples using 'phyloseq' [58] package in R software. To identify the most abundant and indicator sequences BLASTn searches were performed in the National Center of Biotechnology (NCBI) nucleotide database (https://www.ncbi.nlm.nih.gov). Representative sequences for each OTU were aligned to a curated *nifH* alignment [59] using the ARB [60] and its integrated aligner.

Alignments were further checked manually and sequences added to the curated tree using the ARB parsimony tool. The resultant tree was exported from ARB.

Soil chemical characteristics

We used soil data that is described in detail elsewhere [24]. For the purpose of this study, we only used soil data that originated from the 24 plots where soil was collected for this study. In short, between June 11–16, 2012, seven soil samples (one per 2 m × 2 m subplot, taken in its centre) were collected in each of the 10 m × 10 m permanent plots. Samples were taken at 0–20 cm depth using a 50-mm diameter sand auger. Soil samples were sieved (2 mm) after collection. Samples were left to air-dry and chemical analyses were performed by Smithsonian Tropical Research Institute Soils Laboratory. The full list of measured soil variables and methods are summarized in Online resource 1; their means and standard errors by chronosequence stage are presented in Online resource 2.

Statistical analyses

Non-metric dimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix was carried out on subsampled (i.e., rarefied) data generated from the OTU species matrix. This allowed visual inspection to identify compositional differences in rhizobial communities between the five dune stages. Permutational multivariate analysis of variance [61] was used (9999 permutations) to test for differences in symbiotic community composition among dune stages. To estimate alpha diversity within dune stages, we calculated Shannon and Simpson diversity indices on the subsampled matrix. To analyse for differences in beta diversity among chronosequence stages, we used ‘betadisper’ function. To analyse the effect of chronosequence stage on rhizobial diversity and richness, one-way ANOVA was performed. To determine indicator species from each dune stage we used alpha value set on 0.05 and 999 permutations. We performed dbRDA (distance based canonical redundancy analysis) to

assess the effect of soil chemistry on symbiotic community composition using the same dissimilarity matrix as for NMDS. For all soil variables, we first performed a forward selection using the vegan 'ordistep' function with 9999 permutations to identify the environmental variables significantly explaining variation in community composition. We then tested estimated variance inflation factors (VIF) for all variables and excluded variables with VIF >3 from the analysis to minimise collinearity effects [62]. Summaries of redundancy analysis (dbRDA) statistics for five redundancy axes are presented in Online Resource 3. Additionally, a summary of permutation test for dbRDA under the reduced model with 999 permutations for five abiotic variables tested in the dbRDA analysis is presented in Online Resource 4. All analyses were performed in R (R Core Team 2017), using the 'vegan' [63], 'indicspecies' [64], 'MASS' [65], 'ggplot2' [66], 'Rmisc' [67], 'lsr' [68], 'phyloseq' [58] and 'jurien' [30] packages.

Results

From 925,629 *nifH* sequences, we identified 500 OTUs from *A. rostellifera* nodules. After removing singletons and subsampling, 335 OTUs remained (for a total of 867,291sequences). We found 12 OTUs that were present in nodules from all chronosequence stages. These 12 OTUs included three OTUs that are members of *Bradyrhizobium sp*., one *Rhizobium sp*., one *Sinorhizobioum sp*. (*Ensifer sp.),* one *Mesorhizobium sp.* and one *Paenibacillus sp.* (Table 2). Three *Bradyrhizobium sp.* that were found in all samples comprised 40 % of all original sequences (i.e. 373, 350 sequences) (Table 2). There was an overlap in a number of abundant rhizobial taxa between younger and medium old soils with notable differences in abundant rhizobial taxa in the oldest chronosequence stage compared to medium old soil stages (Fig.1). The phylogenetic analysis showed three distinct *nifH* clusters (Online resource 5). The twelve most common OTUs were spread across three clusters.

There were significant differences between dune stages in the multivariate dispersions ($F_{(4,19)}$ = 4.41, P = 0.011). Notably, the rhizobial communities found in stage 3 had significantly lower dispersion (i.e., lower variation in rhizobial composition among replicates) compared to the other chronosequence stages, whereas the rhizobial communities in the stage 5 had significantly higher dispersion (Fig. 2A). Dispersion in rhizobial communities in stage 1 was significantly higher compared to stage 2 (P_{perm} = 0.013) and stage 3 (P_{perm} = 0.002). Dispersion in rhizobial communities in the stage 4 was significantly higher compared to the stage 3 (P_{perm} = 0.018) and higher compared to the stage 2 (P_{perm} = 0.046).

Rhizobial composition in nodules differed significantly among chronosequence stages ($F_{(4,19)}$ = 2.67, P = 0.001). Rhizobial communities from the stages 1, 2 and 3 grouped closer together, whereas rhizobial communities from the stage 5 were farther apart and marginally clustered away from rhizobial communities from other stages (Fig. 2B).

Our dbRDA model was statistically significant ($F_{(5,18)}$ = 2.081, P = 0.001; Fig. 3). This model explained 19.02 % (R^2_{adj}) of the overall variation in the community composition, with dbRDA axes 1 and 2 accounting for 13.1 % and 2.9 % of this effect, respectively. Axis 1 was statistically significant, whereas Axis 2 was not significant (Online resource 3). Soil pH, exchangeable manganese (exchMn), readily-exchangeable phosphate (resinP), nitrate (NO_3) and rate of nitrification (NO_3incub) explained best the rhizobial variation along the Axis 1 across five dune systems (Fig. 3). However, the permutation test for five abiotic variables tested in the dbRDA analysis revealed that these factors were not significant (Online resource 4) suggesting that a combination of abiotic factors rather than any single tested factor explains best the overall effects on bacterial composition.

Indicator species analysis revealed that one OTU was strongly and significantly associated with the stage 3 (Table 3). Two OTUs were strongly associated with the stage 4, one of

which was closely identified as *Tolypothrix sp.* (Table 3). Notably, 28 OTUs were significantly associated with the stage 5. Of these 28 OTUs, 19 were uncultured taxa, four were different members of the *Bradyrhizobium* genus, three were *Tolypothrix sp.*, two *Burkholderia sp*., one was identified as *Clostsridium pasteurianum*, *Chlorobium phaeovibrioides* and *Paenibacillus azotofixans* (Table 3). In the phylogenetic tree, the 28 OTUs from dune stage 5 were from clusters 2 and 3 (Online resource 5).

In addition, there were some OTUs whose patterns of occurrence were more associated with several dune stages (Table 3). For instance, five OTUs were strongly associated with both stages 4 and 5. The most common shared taxa were *Bradyrhziobium* species (Table 3).

Despite differences in community composition, there were no significant differences among chronosequence stages in rhizobial OTU richness ($F_{(4,19)} = 1.47$, $P = 0.25$), Shannon ($F_{(4,19)} = 1.43$, $P = 0.26$) and Simpson diversity indices ($F_{(4,19)} = 0.69$, $P = 0.60$). Rarefaction curve reached an asymptote suggesting that diversity has been saturated (Online resource 6).

Discussion

Our study provides the first characterisation in symbiotic community composition and diversity in nodules across the Jurien Bay chronosequence. This chronsequence is one of the strongest local, natural nutrient-availability gradients ever characterised; showing a 60-fold change in total soil P over 15 km [24]. For example, the total P in our soils ranged from 432 to 6.6 mg kg^{-1} (Table 1). As comparison, the global average of soil P has been estimated to be 700 mg kg^{-1} for continental crust [69]. In contrast, in chronosequences elsewhere, e.g., Cooloola chronosequence in subtropical Queensland (Australia), total P ranged from 229–237 kg ha^{-1} in the youngest soils (40 years old) to 24–28 kg ha^{-1} on the oldest soils (195–>

460 ka) [70]. By holding the trapping host species identity constant, we showed that there is strong variation in rhizobial communities across this chronosequence reflecting pedogenesis-driven changes in soil chemistry. Despite the changes in community composition, there was a large number of abundant rhizobial taxa throughout the chronosequence.

Putative N_2-fixing legumes are abundant throughout the entire chronosequence, with the dominant legumes, in addition to *A. rostellifera,* being *A. lasiocarpa, A. pulchella var. glaberrima*, *Acacia spathulifolia, A. truncata*, *A. xanthina, Bossiaea eriocarpa, Hardenbergia comptoniana, Jacksonia floribunda, J. hakeoides* and *Labichea cassioides* [47,37,71]. *Acacia* is present in all chronosequence stages, especially stages 1 to 3, whereas in stages 4 to 5 other legume genera dominate [37]. In particular, our study highlighted the occurrence of a unique set of N_2-fixing bacteria in *A. rostellifera* nodules in the oldest dune stage that are likely adapted to extreme P limitation. However, whether this observed pattern is an indicator of adaptation to extreme P limitation directly or indirectly through host specificity is yet to be confirmed. The oldest chronosequence stage was dominated by other legumes than *A. rostellifera*, which could partially explain the strong nodule rhizobial compositional differences observed in our trapping experiment, possibly driven by different legume communities that conditioned the field collected soil rather than direct effects of soil abiotic conditions on rhizobia *per se*. Taken together, the role of the overlying plant community is implicit in our models, but not explicit. Thus the exact role of the edaphic changes (direct or indirect) is unknown, whereas it is clear that edaphic conditions have a strong effect on the rhizobial communities in our study system.

There were large changes in rhizobial composition in *A. rostellifera* nodules across the chronosequence, suggesting that environmental heterogeneity and associated nutrient variability is an important driver of rhizobial composition. As such, our results agree with

those of previous studies exploring shifts in mycorrhizal fungal communities in *A. rostellifera* roots and soil along the Jurien Bay chronosequence [23,21]. In chronosequence systems elsewhere e.g., The Franz Josef chronosequence in southern New Zealand, a strong niche differentiation in AM fungal communities with different OTUs being unique to early succession stage, ecosystem progression and ecosystem retrogression was found [29]. These results suggest that at least root mutualistic endophyte composition is similarly driven by environmental heterogeneity along chronosequence stages.

Soil N and P levels, as well as water availability, have been reported to affect all stages of nodule formation, N_2 fixation and rhizobial community structure [72,8,19]. In our study system, there are considerable shifts in N, P and pH (Table 1) across the chronosequence, reflecting pedogenic changes. We found that nitrate and rate of nitrification, pH, readily exchangeable phosphate and exchangeable manganese affected the nodule rhizobial composition across the five chronosequence stages. These results support previous studies that have reported strong effects of soil abiotic conditions on rhizobial diversity and composition [74,19,17].

Mutualism theory suggests the value of a traded resource decreases as its external availability increases [75,13]. Therefore, it has been proposed that as soil N increases, it will favour plant selectivity of rhizobium partners [8]. Alternatively, high soil N availability could also facilitate proliferation of less symbiotically effective rhizobia [4]. In our study system, total N was lowest in youngest and oldest soils and highest in intermediate-aged soils, whereas in intermediate-aged soils (i.e., stages 2, 3) soil [P] was also high, suggesting that rhizobia would not be limited by P and would rather be controlled by N availability in these intermediate-aged soils. However, despite these shifts in N and P levels in the soil, the diversity remained overall similar in the nodules, dominated by *Bradyrhizobium sp*.,

suggesting that host-species led effects may over ride abiotic soil conditions. It is important to note, that we did not assess the *nifH* based rhizobial diversity across the five chronosequence stage soils where there may be apparent changes that our trapping host species' nodules did not detect. We only assessed the rhizobial diversity in the nodules which are likely a subset of rhizobial community in the soil and may be more reflective of the specific relationship between the host plant species and its symbiotic relationships rather than the true alpha diversity of N_2-fixing bacteria in these soils.

Resource trade-off theory postulated that the traits that may promote greater fitness in one environment may lead to a poor performance in another [77,78] and thus lead to specialization for either or both. Soil pH levels are known to exert a very strong selective pressure on rhizobial diversity, with particular conditions favouring rhizobial strains adapted to specific pH which may, then, dominate populations and colonize plant nodules more readily within specific pH zones [79-81]. Some studies have shown that a soil pH <4.5 can be detrimental for the two most common rhizobial genera, i.e. *Rhizobium* and *Bradyrhizobium* [44]. However, in our study system, the soil pH levels ranged from 8.2 to 4.8 and we found both genera, along with *Ensifer sp*., to be highly abundant across all nodules that were harvested from five different chronosequence stages, suggesting that soil pH levels did not affect at least the common rhizobia (*Rhizobium, Bradyrhizobium and Ensifer*) in these soils. The presence of these three common genera in *A. rostellifera* nodules is consistent with findings from *A. rostellifera* rhizobial communities in other parts of Western Australia [52] and other *Acacia* species in Australia [51]. Nevertheless, our dbRDA analysis did reveal that pH affected the rhizobial nodule composition in dune stages 1 to 3, where average pH was above 7.5 as compared to stages 4 to 5 where pH was below 6. This suggests that rhizobial composition of less abundant strains may be filtered in nodules based on soil pH levels, since

prior to infecting hosts rhizobia exist as free-living soil inhabitants directly affected by soil abiotic conditions.

Studies have shown that phylogenetic richness of rhizobial populations is considerably higher on host root surfaces than those found in the nodules [82,83]. Even for promiscuous host/symbiont partnerships certain rhizobial genotypes are more likely than others to adsorb and infect specific host species (i.e., phylogenetic clustering) [84,85]. For instance, different species of *Lotus* formed nodules with a small subset of genetically more related rhizobia that were available to it in its zone near the root [83]. The observed dominance of a few common rhizobia genera in the nodules across all soils found in our study supports the observations by others [83,84,86]. The prevalence of several abundant genotypes of *Bradyrhizobium* in our study is consistent with previous studies on Australian *Acacia* species [79,51]. Notably, we also found that disproportionally more, i.e., 28 phylogenetically diverse OTUs, contributed significantly to the oldest soils. A large number of unclassified OTUs in severely P-impoverished soils suggest diverse rhizobial communities adapted to extreme P limitation which warrants further research.

Conclusions

In summary, we found major shifts in the composition, but not diversity, of rhizobial communities of *A. rostellifera* nodules across five contrasting stages of the Jurien Bay dune chronosequence whose ages ranged from 0.05 ka to > 2000 ka old. These changes in composition were linked to strong shifts in pH, soil N and P concentrations and reflect changes in the host-rhizobia interaction and changes in abundances of a subset of rhizobial strains along the soil nutrient availability gradient. This study contributes to improved understanding of the ecological and evolutionary outcomes of rhizobia-legume interactions in natural systems along a strong environmental gradient. Long-term fspecaccum

soil chronosequences, such as the one used in our study, are valuable natural systems where the effects of soil fertility on plant-mutualism interactions can be robustly tested within bounds of ecological relevance [22]. Future studies are needed that evaluate the functional outcomes of these symbioses across the chronosequence gradient and their role in structuring plant communities.

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Author contributions

FPT and EL designed the experiment. FPT supervised and maintained the experiment. CB conducted the DNA extractions. CB performed statistical analyses with assistance from all authors. AB conducted the bioinformatics work. CB led the writing of the manuscript and all authors contributed to revisions.

653 **Table 1.** Chronosequence stages, estimated soil ages, most likely plant-growth limiting nutrients based on Laliberté *et al*. (2012). Names of
654 dune systems are based on McArthur & Bettenay (1974). Values for soil chemical characteristics are means ± standard errors (SE), n = 70.

655

Chronosequence stage	Dyne system	Estimated soil age (ka)	(Co-) limiting nutrient (s)	Total N (g kg -1)	Total P (mg kg $^{-1}$)	pH ($CaCl_2$)
1	Quindalup (young)	0.05 (Holocene)	N	0.51 ± 0.01	350.9 ± 2.4	8.15 ± 0.01
2	Quindalup (medium)	1 (Holocene)	N, P and/or other nutrients	1.16 ± 0.01	432.2 ± 4.8	7.84 ± 0.01
3	Quindalup (old)	6.7 (Holocene)	N, P and/or other nutrients	0.95 ± 0.05	285.8 ± 13.5	7.77 ± 0.01
4	Spearwood	125 (Middle Pleistocene)	P	0.28 ± 0.01	20.3 ± 0.5	5.77 ± 0.03
5	Bassendean	> 2000 (Early Pleistocene to Late Pliocene)	P	0.24 ± 0.01	6.6 ± 0.2	4.75 ± 0.03

656 **Table 2**. Twelve OTUs out of 335 OTUs that were detected across all chronosequence stages in *A. rostellifera* nodules. Sequence names
657 determined following NCBI nucleotide BLAST search. GenBank accession numbers to closest match are given. *Sinorhizobium sp. = Ensifer sp.*
658 In total, these sequences comprised 621,219 of raw reads or 67% of all raw sequences.

659

OTU number	**Accession number**	**Name**	**Number of raw reads**
OTU1	FJ348670	*Bradyrhizobium sp.*	290,564
OTU2	JQ362368	*Sinorhizobium sp.*	150,169
OTU5	JQ362368	*Sinorhizobium sp.*	79,778
OTU4	HQ259543	*Bradyrhizobium sp.*	44,876
OTU14	HQ259543	*Bradyrhizobium sp.*	37,910
OTU37	DQ098170	*Uncultured nitrogen-fixing bacterium*	5,397
OTU21	EU130424	*Mesorhizobium sp.*	5,330
OTU56	FJ807383	*Uncultured bacterium*	2,433
OTU34	AJ888985	*Paenibacillus sp.*	2,050
OTU52	AJ871103	*Uncultured nitrogen-fixing bacterium*	1,099
OTU43	FJ008582	*Uncultured soil bacterium*	918
OTU49	AY934876	*Rhizobium hainanense*	695

661 **Table 3.** Indicator species analysis (alpha = 0.05) output showing OTUs detected in *A.*
662 *rostellifera* nodules that contributed significantly to different soil stages as well as their
663 combinations (i.e. shared OTUs). Names determined following NCBI nucleotide BLAST
664 search. GenBank accession numbers to closest match are given.

665

OTU number	Indicator value	*P* value	Chronosequence stage	Accession number	Name
OTU19	0.773	0.049	3	DQ520544	Uncultured bacterium
OTU400	0.755	0.048	4	DQ078036	Uncultured bacterium
OTU176	0.738	0.032	4	AY768421	*Tolypothrix sp*.
OTU163	0.843	0.011	5	CP010313	*Bradyrhizobium japonicum*
OTU485	0.798	0.012	5	GQ241888	Uncultured microorganism
OTU192	0.778	0.013	5	DQ520450	Uncultured bacterium
OTU293	0.772	0.025	5	AJ567343	*Burkholderia vietnamiensis*
OTU124	0.707	0.027	5	FJ008542	Uncultured soil bacterium
OTU127	0.707	0.027	5	GQ441409	Uncultured bacterium
OTU140	0.707	0.027	5	CP000607	*Chlorobium phaeovibrioides*
OTU164	0.707	0.027	5	AP012279	*Bradyrhizobium sp*.
OTU191	0.707	0.027	5	DQ177006	Uncultured bacterium
OTU269	0.707	0.027	5	AY601073	Uncultured bacterium
OTU315	0.707	0.027	5	DQ098217	Uncultured nitrogen-fixing bacterium
OTU359	0.707	0.027	5	HM210393	Uncultured bacterium
OTU377	0.707	0.027	5	DQ776672	Uncultured soil bacterium
OTU381	0.707	0.021	5	AJ299453	*Paenibacillus azotofixans*
OTU391	0.707	0.027	5	AY768421	*Tolypothrix sp*.
OTU397	0.707	0.021	5	EU594068	Uncultured bacterium
OTU419	0.707	0.027	5	EF158806	*Burkholderia caryophylli*
OTU420	0.707	0.021	5	JF897505	Uncultured bacterium
OTU426	0.707	0.027	5	AB011895	Unidentified nitrogen-fixing bacteria
OTU436	0.707	0.021	5	DQ304830	Uncultured bacterium
OTU437	0.707	0.027	5	FJ233712	Uncultured prokaryote clone
OTU441	0.707	0.021	5	EF394168	*Bradyrhizobium japonicum*
OTU481	0.707	0.027	5	AY768421	*Tolypothrix sp*.
OTU483	0.707	0.027	5	GQ441592	Uncultured bacterium

OTU497	0.707	0.027	5	CP010313	*Bradyrhizobium japonicum*
OTU286	0.696	0.049	5	EU622628	Uncultured bacterium
OTU463	0.667	0.043	5	EF568474	Uncultured microorganism
OTU464	0.657	0.045	5	AY603957	*Clostsridium pasteurianum*
Shared OTUs					
OTU67	0.949	0.001	1+2	AB273228	Uncultured nitrogen-fixing bacterium
OTU358	0.999	0.015	1+5	HQ259543	*Bradyrhizobium sp.*
OTU295	0.784	0.02	1+5	AP012048	*Arcobacter sp*.
OTU247	0.775	0.021	2+3	EU252609	*Bradyrhizobium elkanii*
OTU31	0.995	0.004	4+5	CP002737	*Methanotorris igneus*
OTU30	0.941	0.008	4+5	HQ259563	*Bradyrhizobium sp*.
OTU132	0.825	0.043	4+5	EU048063	Uncultured bacterium
OTU23	0.816	0.023	4+5	DQ520522	Uncultured bacterium
OTU44	0.816	0.013	4+5	FJ008205	Uncultured soil bacterium
OTU103	0.96	0.001	1+2+3	FJ008332	Uncultured soil bacterium
OTU6	1	0.006	1+4+5	HQ259543	*Bradyrhizobium sp*.
OTU70	0.963	0.001	1+4+5	HQ259543	*Bradyrhizobium sp.*
OTU346	0.865	0.02	1+4+5	AY768421	*Tolypothrix sp*.
OTU10	1	0.001	1+2+4+5	HQ259543	*Bradyrhizobium sp*.
OTU42	1	0.018	1+3+4+5	HQ259543	*Bradyrhizobium sp*.

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679 **Figures**

680 **Figure 1.** Heatmap of 335 OTUs per chronosequence stage based on *nifH* classification. The
681 darker the shade the more abundant an OTU was.

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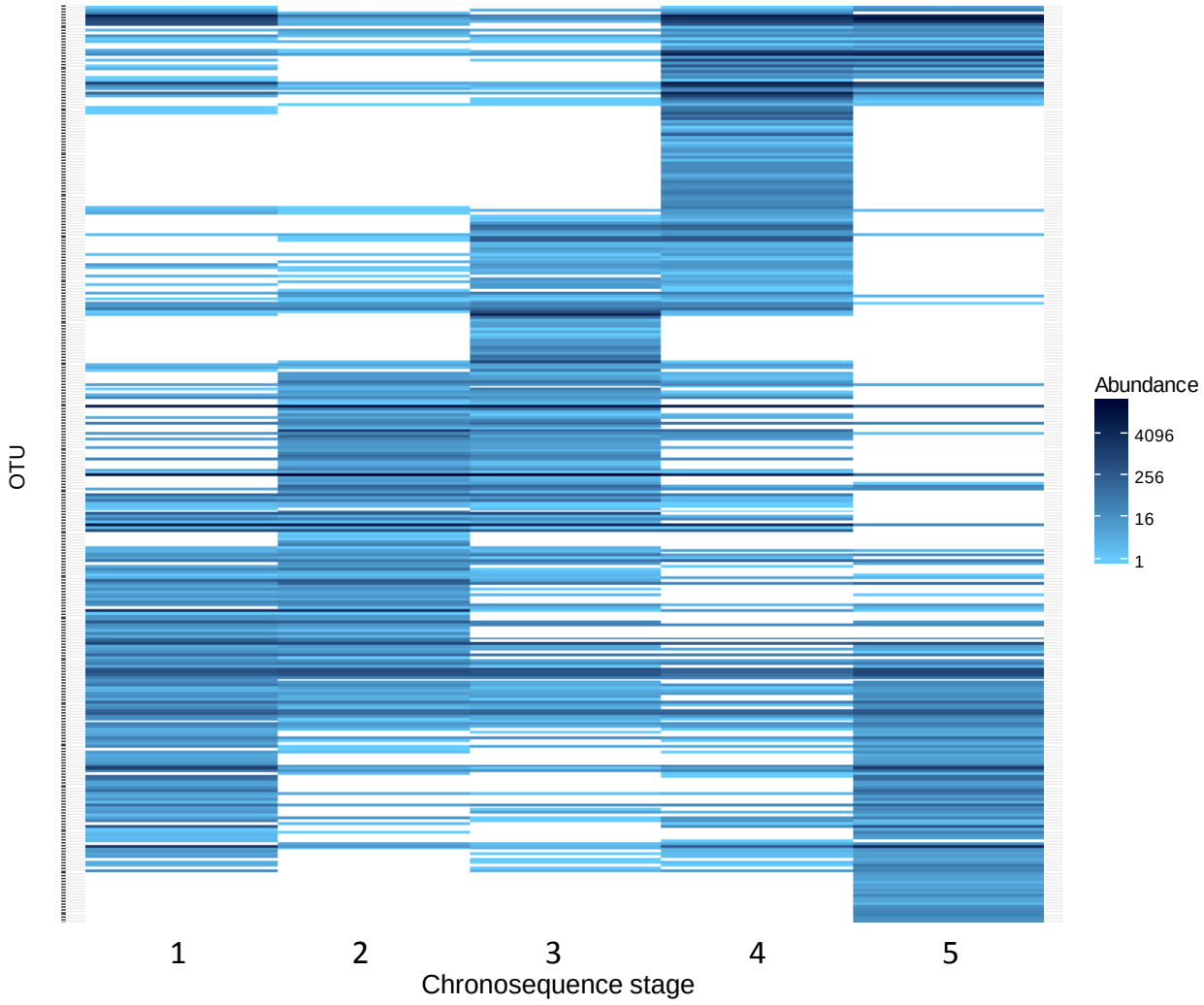

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Figure 2. A - Distances to centroid and average distance to median (numbers) for rhizobial communities based on extracted DNA from *Acacia rostellifera* nodules and amplified *nifH* gene based on soil collected from five progressive chronosequence stages in Jurien Bay, Western Australia. B - Non-metric multidimensional scaling ordination for rhizobial communities based on extracted DNA from *Acacia rostellifera* nodules and amplified *nifH* gene based on soil collected from five progressive chronosequence stages in Jurien Bay, Western Australia.

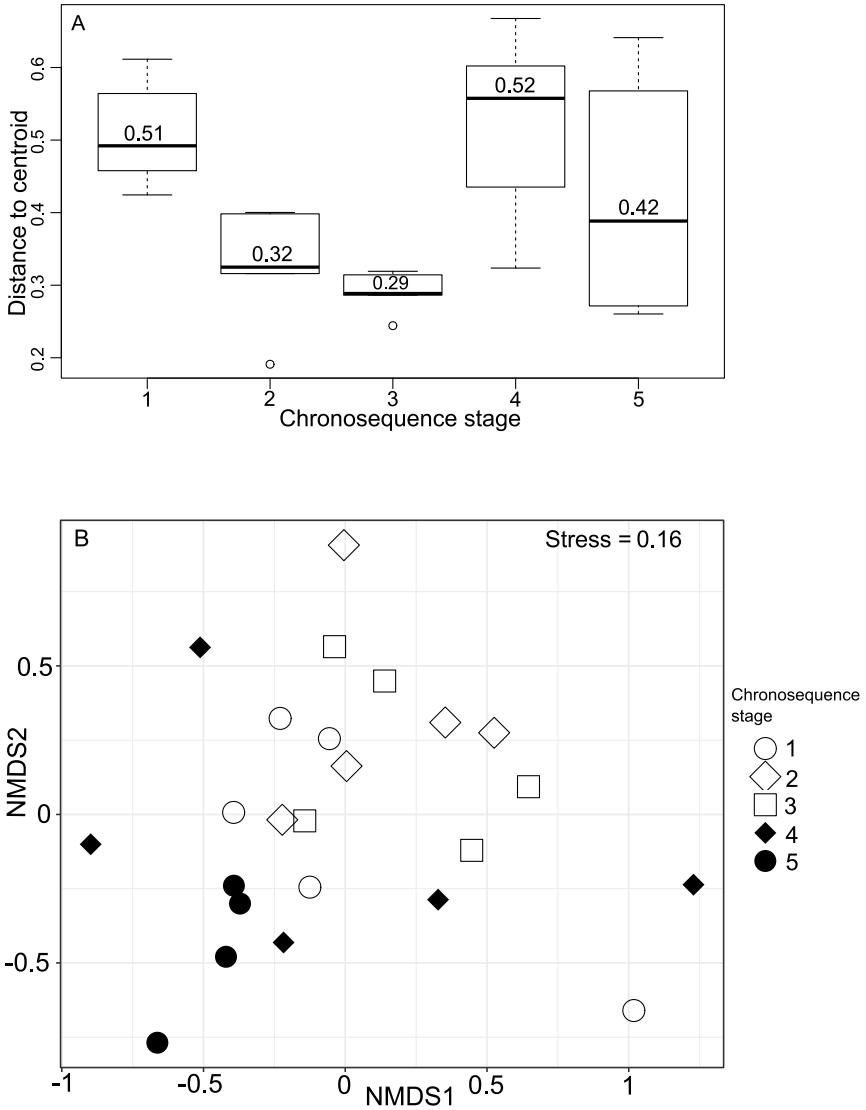
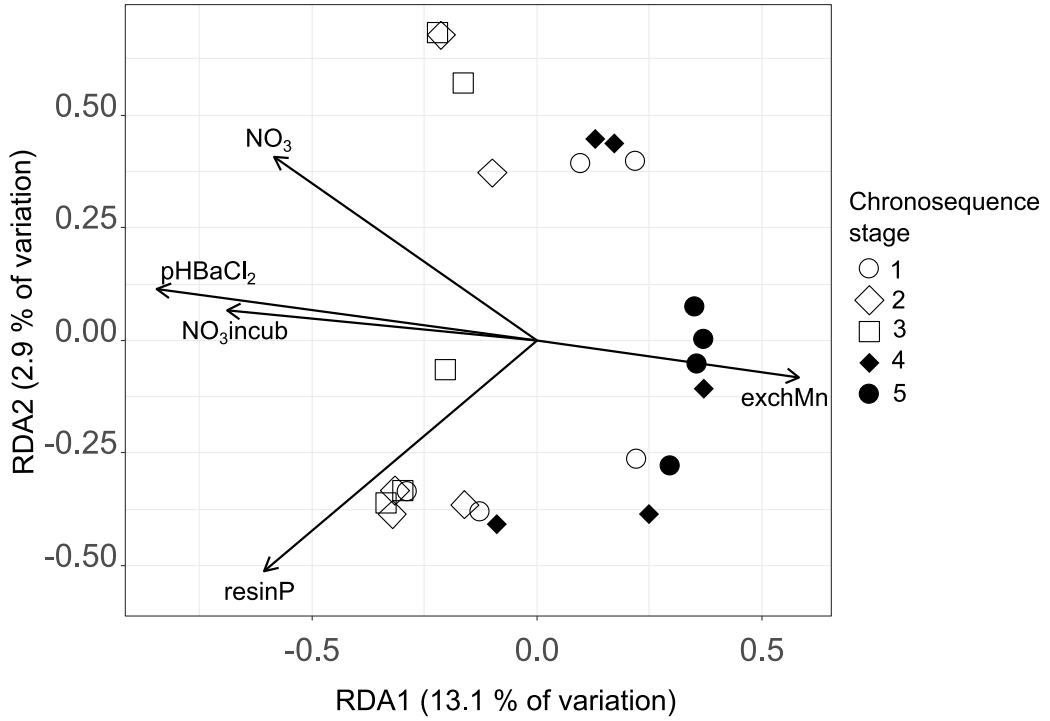


Figure 3. dbRDA biplot for rhizobial communities based on extracted DNA from *Acacia rostellifera* nodules and amplified *nifH* gene based on soil collected from five chronosequence stages in Jurien Bay, Western Australia.



Supplementary material

Symbiotic N_2-fixer Community Composition, but Not Diversity, Shifts in Nodules of a Single Host Legume Across a 2-million-year Dune Chronosequence

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Running title: Rhizobial communities and ecosystem development

Online resource 1. List of 30 soil variables, sampling levels and methods used in dbRDA analyses. (Separate Excel file)

Online resource 2. Means and ±S.E. of 30 soil variables used in the dbRDA analysis. (Separate Excel file)

Online resource 3. Summary of redundancy analysis (dbRDA) of the effects of environmental variables on rhizobial composition across five chronosequence stages in Jurien Bay, Western Australia. Summary statistics for five redundancy axes are presented. PVE = percentage constrained variation explained.

		PVE				
RDA axis	**Eigenvalue**	**Unconstrained**	**Constrained %**	***F***	**Df num, den**	***P***
Axis 1	0.017	0.012	13.1	7.177	1,18	0.001
Axis 2	0.004	0.007	2.9	1.612	1,18	0.647
Axis 3	0.002	0.004	1.3	0.694	1,18	0.995
Axis 4	0.001	0.004	1.1	0.609	1,18	0.985
Axis 5	0.001	0.003	0.6	0.311	1,18	0.997

Online resource 4. Summary of permutation test for dbRDA under reduced model with 999 permutations for five abiotic variables tested in the dbRDA analysis.

Variable	**Df**	**Variance**	***F***	***P***
phBaCl$_2$	1	0.002	1.199	0.271
NO$_3$incub	1	0.003	1.506	0.140
exchMn	1	0.003	1.272	0.218
resinP	1	0.004	1.571	0.114
NO$_3$	1	0.002	0.918	0.487
Residual	18	0.043		

Online resource 5. Phylogenetic tree showing the 335 OTUs based on *nifH* gene amplified from *A. rostellifera* nodules' DNA after growing in the greenhouse in soils collected across five chronosequence stages in Jurien Bay, Western Australia. The tree is based on the neighbour joining tree of Gaby *et al.* (2014) to which 335 OTU sequences were added using the ARB parsimony tool. Three phylogenetically distinct clusters are shown. In blue are marked the 12 most common OTUs based on Table 2. In green are marked OTUs that associated significantly with dune stage 5 (the oldest) based on Table 3. (attached separately)

Online resource 6. Rarefaction curve using "specaccum" function n "vegan" that reached an asymptote, i.e. diversity has been saturated.