# *Original Article*

# **Strong host modulation of rhizosphere-to-endosphere colonisation in natural populations of the pan-palaeotropical keystone grass species, *Themeda triandra***

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**ABSTRACT**

Soil microbiota can colonise plant roots via a two-step selection process, which involves the recruitment of microbiota first from bulk soil into plant rhizospheres, then into root endospheres. This process is poorly understood in all but a few model species, which is surprising given its fundamental role in plant and soil ecology. Here we examined the microbial assembly processes across the rhizospheres and root endospheres in eight natural populations of the pan-palaeotropical C4 grass, *Themeda triandra*, in southern Australia. We assessed whether root endosphere colonisation patterns aligned with the two step-selection process. We also assessed the degree to which the assembly patterns of these rhizospheres and endospheres were influenced by deterministic processes. We show that two-step selection was the dominant recruitment dynamic across these natural *T. triandra* populations, and present clear evidence the host plants influenced microbial assembly via deterministic pressures that produced strong convergence of endospheres. Both endospheres and rhizospheres were influenced by local environmental filtering, including aridity. Our study improves our understanding of assembly processes for root endospheres, central to plant-soil interactions yet poorly understood in non-model species. We show that endospheres of native populations of a widely distributed, keystone grass (*T. triandra*) were strongly shaped by the plant host and displayed patterns consistent with the two-step selection process. These findings raise intriguing questions about the functions of this ‘core’ microbial endosphere, but our limited understanding of their ecology hinders our ability to harness these important relationships to, for example, improve plant propagation and revegetation practices.

**KEYWORDS**

endosphere, microbial ecology, neutral theory model, rhizosphere, *Themeda triandra*, two-step selection process

**INTRODUCTION**

Soil microbiota have important roles in ecosystem functioning as they help to drive ecological processes (e.g., nutrient cycling) and make important contributors to plant growth and fitness (David et al. 2019, Wang et al. 2019, Choi et al. 2021). These soil microbiota commonly interact with plants via plant-soil feedbacks, where plants release organic exudates into the soil via their roots which then influence microbial community structure and diversity patterns (Bever et al. 2010). In turn, microbiota can provide their plant hosts with essential nutrients, protection against pathogens, and growth or fitness advantages via the release of metabolites and/or hormones (de Vries et al. 2020, Thiergart et al. 2020, Yang et al. 2021). Though generally poorly understood in non-model systems, a better understanding of these plant-soil feedbacks has promise to help ecosystem managers make more informed decisions about how to introduce or promote plant species (Breed et al. 2019, de Vries et al. 2020, Thiergart et al. 2020), especially during plant propagation, translocation and revegetation efforts (Peixoto et al. 2022, Robinson et al. 2023).

Soil microbiota can colonise plant roots via a two-step selection process, where certain soil microbiota are selectively recruited from bulk soil into plant rhizospheres (the soil and associated microbiota surrounding roots), and then into the root endosphere (the microbiota inside roots) via plant regulation processes (Lundberg et al. 2012, Bulgarelli et al. 2013, Urbina et al. 2018). This two-step selection process is promoted by the deposition of cells and organic exudates that attract microbiota into rhizospheres from bulk soils. From the rhizospheres, microbiota can enter into plant roots to form root endospheres via host-plant genotype interactions and regulations through plant immune systems (Bulgarelli et al. 2013). The differentiation in beneficial microbiota observed across plant rhizospheres and endospheres can be linked to how microbiota are selected by their host plants (Urbina et al. 2018, Stopnisek and Shade 2021). However, the assembly dynamics responsible for the microbial composition of rhizospheres and root endospheres are poorly explored, especially in non-model organisms *in situ* (Naylor and Coleman-Derr 2018, Sasse et al. 2018, Thiergart et al. 2020). Indeed, in natural systems, we might expect assembly processes to depend strongly on local environmental conditions, such as site-level aridity but this is poorly understood (Petipas et al. 2017, Hodgson et al. 2024).

Rhizospheres and endospheres can impact on host plant fitness (Zhang et al. 2020, Durán et al. 2022, Ling et al. 2022). Indeed, it is not only the most abundant microbial taxa that are important for plants; rare microbial taxa can also promote plant health and affect microbial community dynamics (Jousset et al. 2017, Neu et al. 2021, Custer et al. 2023). Identifying rare and abundant taxa, and taxa whose abundances are variable across ecological contexts (i.e., conditionally rare and/or abundant), can provide insight into rhizosphere and endosphere recruitment dynamics (Logares et al. 2014, Xue et al. 2018, Zhang et al. 2018). Highly diverse recruitment strategies can highlight the importance of microbiota fulfilling multiple functions for their hosts, offering long term protection against stress or disturbance through generating functional redundancy (Naeem et al. 1994, Louca et al. 2018). Therefore, characterising the structure of microbial communities – plus microbial taxa that are selected for by plant hosts – can identify functionally important microbial taxa, plus the recruitment strategies used by the host plants (Hamonts et al. 2018 , Risely 2020, Ling et al. 2022).

Neutral ecological theory has been used to ascribe deterministic or stochastic assembly processes to individual taxa based on their abundance patterns (Ofiţeru et al. 2010, Stopnisek and Shade 2021). While these models have assumptions of functional equivalence among taxa and the prevailing nature of stochastic processes (Zhou and Ning 2017, Rocha 2018), they do enable direct comparisons of microbial community assemblies that can detect persistently present taxa that are deterministically selected for in a given plant-soil environment (Burns et al. 2016, Stopnisek and Shade 2021).

*Themeda triandra* is a pan-palaeotropical C4 grass species that is dominant in many grassland ecosystems, worldwide (Snyman et al. 2013). While this plant is widely distributed, grasslands are in global decline (Murphy et al. 2016, Bardgett et al. 2021), and there is a need to build new knowledge that assists its return to ecosystems that are resilient to climate change (Gopal and Gupta 2016, Brinkman et al. 2017, Larson et al. 2022). *T. triandra* is known to strongly associate with its soil microbiota (Hodgson et al. 2024), which can aid its growth and fitness (Hassen and Labuschagne 2010, Petipas et al. 2017). Microbial communities linked to *T. triandra* fitness may also be susceptible to climate change impacts, including warming temperatures, increased CO2 and desertification (Hayden et al. 2012, Tang et al. 2021). Therefore, further understanding the composition of the microbial communities that directly interact with *T. triandra* root structures – such as those surrounding (i.e., rhizospheres) and within (i.e., endospheres) roots – across a diversity of climatic and soil conditions is a key step for identifying the microbial taxa and environmental circumstances that should promote the growth and fitness of this plant (Hayden et al. 2012, Snyman et al. 2013, Gonzalez et al. 2018).

Here we examined the two-step selection process of *T. triandra* through a microbial community assembly lens and assessed how host, climatic and environmental variables shaped *T. triandra* rhizospheres and endospheres. We used 16S rRNA amplicon sequencing to characterise the bacterial rhizospheres and root endospheres to focus specifically on the second step of the two-step selection process (i.e., from rhizospheres into endospheres; our previous work (Hodgson et al. 2024) examined the first step – bulk soil microbiota into rhizospheres). We posed the following research questions: (1) Does *T. triandra* display rhizosphere and root endosphere bacterial community compositions that are consistent with the two-step selection process? (2) Is there evidence of deterministic assembly processes influencing these bacterial communities? And, (3) are bacteria in *T. triandra* rhizospheres constraining endosphere recruitment? These questions were posed to examine whether bacteria contained within the root endospheres of *T. triandra* were predominantly a product of the diversity of rhizospheres or whether specific taxa were recruited by the plant host.

### METHODS

*Study species*

*Themeda triandra* (Forssk.) is a pan-palaeotropical C4 grass species (Dunning et al. 2017). As a keystone species, *T. triandra* is important for supporting invertebrate communities across stable environments (Snyman et al. 2013), and it has important associations with fire – for instance, it deposits flammable leaf litter that accumulate during growth, and its seeds respond well to smoke and high temperatures (Baxter et al. 1994, Ghebrehiwot et al. 2012). *T. triandra* is a difficult species to cultivate, as the seed has been known to germinate best after long dormancy periods, with substantial variation across regions (Saleem et al. 2009, Farley et al. 2013, Hancock and Hughes 2014).

*Field experiment*

In December 2021, soil and plant tissue samples were collected from six *T. triandra* individuals across eight sites along an aridity gradient in southern Australia (aridity index values 0.318–0.903), as described in Hodgson et al. (2024) (Table 1; Figure 1a). There was no correlation between pairwise geographic distances and aridity differences between sites (*p* = 0.489; r = -0.021). Mean annual aridity index data (annual precipitation/annual potential evaporation) was obtained from the Atlas of living Australia (Belbin 2011, ALA 2014) spatial portal, using the Aridity index layer (UNEP 1992, Middleton and Thomas 1997).

The six *T. triandra* plants were sampled from within a 25 x 25 m area at each study site using coordinates generated from a random number table, where for each coordinate the nearest plant was sampled. We profiled the diversity and community composition of bacteria in the rhizospheres and root endospheres of these plants using 16S rRNA amplicon sequencing (described below).

*Isolation and extraction of rhizosphere and endosphere DNA*

Microbial DNA from the rhizospheres was obtained following the protocol outlined in McPherson et al. (2018) and detailed in Hodgson et al. (2024). Briefly, sampled roots were washed in 0.02% Silwet L-77 amended PBS buffer and vortexed, before being filtered at 100 µm and centrifuged, prior to DNA extraction. *T. triandra* endospheres were extracted by removing as many bacteria and DNA as possible from root surfaces and subsequently extracting the DNA directly from these ‘cleaned’ root tissues. To determine the best methods of isolating *T. triandra* endosphere DNA, we ran a pilot study to compare methods of root cleaning via washing, bleaching and sonicating root surfaces (see Supplementary Information for further detail, Figures S1-S3). Based on our pilot study, roots were sonicated on ice in 0.02% Silwet L-77 amended PBS buffer at 30% amplitude for five 30 sec alternating burst and rest periods over 5 minutes. Following this, roots underwent a series of five washes in this sterilised amended PBS buffer solution. Root endosphere samples were pulverised with metal beads for 1 min in bead beating solution (PowerSoil Kit, Qiagen, Hilden, Germany). DNA extractions were then performed on rhizosphere and endosphere samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocols.

*Amplification, sequencing and bioinformatics*

Amplicon libraries of the 16S rRNA V3-4 gene region were developed by the Australian Genome Research Facility (AGRF, Melbourne, Australia). Samples were PCR amplified with the forward primer, 341F (CCTAYGGGRBGCASCAG), and reverse primer, 806R (GGACTACNNGGGTATCTAAT). Sequences were determined using the 300 base pairs paired end run of Illumina MiSeq sequence production. We used the DADA2 bioinformatic pipeline through QIIME2 (Bolyen et al. 2019) to infer identity profiles from amplicon sequence data by matching against the SILVA database (Version 138.1) (Wang et al. 2007, Quast et al. 2013) using a naïve Bayesian classifier (Wang et al. 2007, Callahan et al. 2016). All taxa that were not assigned as Bacteria, and associated to mitochondria or chloroplasts were removed. We also removed taxa that did not occur in at least two samples to avoid nonrepresentative and potentially contaminating taxa.

*Statistics*

All analyses were done in R version 4.0.2 (R Core Team 2022). We rarefied samples to 11,491 reads without replacement to normalise for variation in library size across samples and to maintain a constant sampling effort for downstream analyses (Figure S4). We estimated alpha diversity as effective number of ASVs (eff. no. ASVs.), which were calculated as the exponential transformation of Shannon’s diversity index (Jost 2006). We compared these values across rhizospheres and endospheres using linear mixed-effects models with the *lmer()* function in the lme4 package in R (Bates et al. 2015). Plant compartment (i.e., endosphere, rhizosphere) was treated as a fixed effect, and plant ID was included as a random effect to account for resampling the same individuals across rhizospheres and endospheres. Model significance was tested using a Walk chi-squared test, and pairwise tests were made using multiple comparisons with Tukey contrasts through the *glht()* function of the multcomp package (Hothorn et al. 2008). We also assessed the number of unique taxa within each belowground compartment, and across sites using the Microeco package in R (Liu et al. 2020).

We visualised bacterial communities using non-metric multidimensional scaling ordination (NMDS) with Bray-Curtis distances. Compositional differences between bacterial communities in the endospheres and rhizospheres samples were tested via permutational multivariate analysis of variance (PERMANOVA) using the *adonis2*() function in vegan (Oksanen et al. 2019). We also assessed for homogeneity of group dispersion with vegan’s *betadisper()* function (Oksanen et al. 2019). Visualisations of the relative abundance of the top 11 phyla across treatments were performed using the *plot\_bar*() function in Phyloseq (McMurdie and Holmes 2013). Rare phyla that made up less than 0.5% total relative abundance were grouped as ‘Other minor phyla.’

Neutral theory models

We compared bacterial ASVs found in rhizospheres and endospheres to a neutral model of microbial community assembly to assess host plant selection processes on these taxa (Sloan et al. 2007, Burns et al. 2016, Stopnisek and Shade 2021). We compared distributions of community structure to the Sloan neutral model, which assumes that community structures are principally driven by stochastic processes (i.e., reproduction, mortality, speciation, extinction, colonisation) (Sloan et al. 2007). Although these models can underrepresent taxa or species that are deterministically selected (Stopnisek and Shade 2021), we are still able to use them to hypothesise functionally useful ASVs that may play key roles in *T. triandra* microbiomes. ASVs outside the upper confidence intervals of the neutral model were inferred as those to have undergone positive selection, whereas ASVs outside the lower confidence interval were assumed to have undergone negative selection pressures in that environment.

Conditional abundances of bacterial ASVs

To examine the rarity of bacteria across our dataset, we assigned ASVs to three groups – abundant, moderate and rare – according to whether they met relative abundance thresholds according to Xue et al. (2018). ASVs that had ≥1% relative abundance within their sampling sites were considered abundant taxa (AT), while ASVs with <0.01% relative abundance were considered rare taxa (RT). ASVs between these values (i.e., ≥0.01 but <1%) were considered moderate taxa (MT). Bacterial ASVs that were of relative abundances of ≥0.01% in all sites, but ≥1% in at least one site, were considered conditionally abundant taxa (CAT), whereas those that were found to be of <1% relative abundance in all sites, but <0.01% in at least one site, were conditionally rare taxa (CRT). The ASVs that had instances where relative abundance was at least <0.01% in one site, but ≥1% in another, were considered conditionally rare and abundant taxa (CRAT) The community composition of ASVs in these categories was visualised at the phylum level with chord diagrams using the R package circlize (Gu et al. 2014).

The different abundance categories were then compared against neutral models (described above) to investigate whether these ASVs underwent selection by the host plants. ASVs which were MT, RT, and CRT were examined separately, whereas ASVs that were CRAT and CAT were combined due to the low numbers of taxa in these groupings.

Differentially abundant taxa

We determined differentially abundant taxa across compartments and sites using the R package ANCOMBC (Lin and Peddada 2020) with function *ancombc2*() to reveal phyla and ASVs that were disproportionally present in endospheres versus rhizospheres communities. In this model, plant compartment (i.e., endosphere vs. rhizosphere) was treated as a fixed effect, and plant ID was included as a random effect. We visualised differentially abundant taxa using log-fold changes, maintaining only statistically significant taxa at a 0.05 significance threshold.

We then conducted this analysis again, but for each individual sampling site, identifying differentially abundant taxa using the *ancombc*() function across rhizospheres and endospheres, separately across each of the eight sites. Following software instructions, this differential abundance testing was based on non-rarefied data, and we used the false discovery rate for p-value adjustment for multiple comparisons (Benjamini and Hochberg method) at both phylum-level and ASV-level of our data to identify differing taxa between endospheres and rhizospheres. Comparisons across sites were then made to identify differences in the rhizosphere to endosphere recruitment dynamics within sampling sites.

We then used a three-step approach to explore the neutral and deterministic selection dynamics of the rhizosphere and endosphere differentially abundant ASVs. First, we isolated endosphere and rhizosphere ASVs from our dataset. Secondly, we created lists of ASVs that were disproportionately more abundant in the rhizosphere (= those with significant positive log fold change), those more abundant in the endosphere (= significant negative log fold change), and those that were not differentially abundant (non-significant effect). Finally, we examined neutral assembly models based on these three lists of ASVs, using rarefied data for making model comparisons (as used in our diversity analyses), to separately compare selection in the endospheres and rhizospheres. We used this approach to show how selection on ASVs changed from the rhizosphere to endosphere. Model fits were assessed using the coefficient of determination (R2).

Co-occurrence network analysis

We used co-occurrence network analysis of bacterial ASVs to determine interactions between taxa and derive an indication of community structure within endospheres and rhizospheres. This analysis explores connections between different ASVs (nodes) via their connections (edges) and estimates significant positive or negative associations for these taxa. ASVs were filtered to the number of associations within the communities to give a measure of community complexity and to compare patterns of occurrence of taxa within endospheres and rhizospheres. We used SparCCto define absolute abundance associations between taxa at the ASV level, using the Spiec-Easi R package (Friedman and Alm 2012, Kurtz et al. 2015).

For visualisations, selection rigour and computational processing of the network analyses, we only report ASVs with >100 sequences. Randomly permuted (*n* = 1000) data were used to estimate the statistical significance of associations. Taxon associations were included using SparCC correlations at ≥0.65, with *p* <0.05. We used the R package Matrix (Bates et al. 2023) to create a matrix from the given set of values and igraph (Csardi et al. 2006) to visualise and evaluate the plots. We identified ‘hub’ taxa as the top 30 bacterial ASVs with the highest positive or negative node edges. These taxa likely play a critical role within a community, based on their links to other taxa.

### RESULTS

*Bacterial diversity in belowground compartments*

Across all compartments, we observed 11 bacterial phyla that represented 99.5% of reads and had abundance estimates of >2% (Figure 2a). Rhizospheres and endospheres had strongly different bacterial alpha diversity (LMEM: *X2(1)*= 56.220, *p* <0.001; Figure 1b), and sampling site also had a strong effect (LMEM: *X2*(7)= 24.522, *p* <0.001). Rhizospheres had an effective number of ASVs of 296, compared to 153 in endospheres (*p* <0.001). Additionally, 96% of ASVs were shared between compartments (Figure 3a). Interestingly, 3.6% of taxa were unique to the endospheres (= 1031 ASVs), whereas only 0.3% of taxa were unique to rhizospheres (= 158 ASVs). The endospheres had greater abundance of sequences belonging to the phyla Actinobacteria and Myxococcota and lower Acidobacteria (Figure 3b).

Compartment had a strong effect on bacterial community composition, with clear separation between *T. triandra* endospheres and rhizospheres (Figure 1c; PERMANOVA: F(1, 76*)* = 10.888, R2= 0.078, *p* = 0.001, *n*= 48 samples per group). Communities were tightly clustered by sampling site (Figure S5; PERMANOVA: *R2*= 0.287, F(7,76)= 5.741, *p*= 0.001, *n*= 6 per site), and we found strong evidence of interactions between sampling site and belowground compartment (PERMANOVA: *R2*= 0.092, F(7,76)= 1.839, *p*= 0.001, *n*= 6 per site). We also found a significant relationship between bacterial community composition and site aridity across both the rhizospheres (Figure S6; PERMANOVA: *R2*= 0.065, F(1,42)= 2.914, *p* < 0.001) and endospheres (Figure S6; PERMANOVA: *R2*= 0.064, F(1,46)= 3.147, *p* < 0.001).

Endospheres were less heterogeneous than rhizospheres (ANOVA: F(1,90)=36.24, *p* <0.001; Figure 2d), suggesting overall convergence of these *T. triandra* bacterial communities across sampling sites.

*Taxonomic rarity and abundance*

Bacterial ASVs were delineated into different rarity and abundance categories (see Methods for details; Table 2). Only one ASV was abundant in the endospheres across all samples, and no ASVs were abundant in the rhizosphere (>1% abundance). As a proportion of the whole community, the greatest difference between endospheres and rhizospheres was in the conditionally rare taxa (<1% in all sites, but <0.01% in some), which comprised 62% of bacterial sequences in the endospheres (5,070 ASVs) and 79% of sequences in the rhizospheres (5,705 ASVs; Table 2). The rare taxa (<0.01% in all sites) also showed a large difference between compartments, comprising 37% of sequences in the endospheres (2,970 ASVs) and 21% of sequences in the rhizospheres (1,477 ASVs; Table 2). Across the rhizospheres and endospheres, all ASVs had similar taxonomic compositions at the phylum level across MT, CAT, and CRAT categories (Figure 2b-c). However, we did see a change in the relative number of bacterial sequences in the RT to CRT categories between rhizospheres and endospheres (Figure 2b-c).

*Differentially abundant taxa among endospheres and rhizospheres*

We found 12 bacterial phyla were differentially abundant across the endospheres and rhizospheres using the ANCOM-BC approach (Figure S7a; Table S1). Phyla were predominantly enriched in the rhizosphere and diminished in the endosphere, and included bacteria attributed to: Verrucomicrobiota, WSP2, Chloroflexi, Armatimonadota, RCP2-54, Acidobacteriota, Gemmatimonadota, and Planctomycetota (Figure 2d). The endosphere enriched phyla included: Patescibacteria, Actinobacteriota, Proteobacteria and Myxococcota (Figure 2d). In a separate differential abundance analysis at the ASV level, we found 218 ASVs were differently abundant (Figure 4a; Table S2).

When we performed this analysis separately for at each site, and compared site outcomes together, an average of 8.5 differentially abundant phyla were present across rhizospheres and endospheres (Figure S7b). Specifically looking at directional trends, we found that 4.5 phyla were more abundant in the endospheres (negative log fold changes) and 4 phyla were more abundant in rhizospheres (positive log fold changes; Figure S8; Figure S11).

There were 388 differentially abundant ASVs between endospheres and rhizospheres (Figure S9). When we compared these patterns across sites, we observed 182.1 ASVs were more abundant in endospheres (negative log fold changes) and 217.3 ASVs were more abundant in rhizospheres (positive log fold changes; Figure 4b). Interestingly, the differentially abundant ASVs across rhizosphere and endospheres at the site level were often unique to each site. Only 1 ASV was differentially abundant in every site, whereas a mean of 197 ASVs were uniquely differentially abundant across all sites (Figure 4c). The remaining ASVs were shared across two or more sites in various combinations, with diminishing counts as site-site comparisons became more inclusive (Figure S9-10).

*Selection under neutral theory of community assembly*

Endospheres fitted the neutral model to a lower degree than rhizospheres (Figure 5; endosphere, R2 = 0.317, rhizosphere, R2 = 0.464).

We then applied the neutral model to the different rarity and abundance categories of bacterial ASVs in rhizospheres and endospheres (Figure S12). The abundance patterns of MT and CRT had better fitting neutral models in the rhizospheres (R2 = 0.469, Figure S12a; R2 = 0.49, Figure S12c, respectively) compared to the endospheres (R2 = 0.12, Figure S12e; R2 = 0.424, Figure S12g, respectively). The CRAT+CAT and RT neutral models had poorer fits in rhizospheres (albeit with RT producing a poor fitting model; R2 = 0.295, Figure S12d; R2 = -0.129, indicating failure to fit a model, Figure S12b, respectively), compared to the endospheres (R2 = 0.175, Figure S12f; R2 = 0.466, Figure S12h, respectively).

Comparing the differentially abundant ASVs of the rhizospheres and endospheres also revealed interesting microbial community assembly patterns and selection dynamics (Figure S13). Differentially abundant rhizosphere-favoured ASVs (positive log fold change) displayed a better fit of the neutral models in rhizospheres (R2 = 0.286; Figure S13a) compared to endospheres (negative log fold change) (R2 = 0.014; Figure S13e), indicating that endospheres were under stronger deterministic pressures than rhizospheres. Non-differentially abundant ASVs in the rhizospheres and endospheres both appeared to contain communities with poor fitting neutral models, which suggests that deterministic processes were influencing many of the taxa in these compartments even when there was no evidence of differential abundance differences between them (R2 =0.039, Figures S13c; and R2 =0.028, Figure S13f, respectively). When looking at how the neutral models fitted the endosphere-favoured ASVs (negative log fold change) within the rhizosphere samples, we observed strong neutral influences (R2 = 0.695, Figure S13b); and similarly, the rhizosphere-favoured ASVs (positive log fold change) when found in endosphere samples saw comparatively neutral distribution (R2 = 0.454, Figure S13d).

*Network analysis and hub taxa*

Our network analysis included 1,208 ASVs in rhizospheres and 826 ASVs in endospheres. After filtering to associations between nodes with SparCC correlations ≥0.65 and p-values <0.05, then removing any isolated nodes, we produced network objects with 81 nodes (= ASVs) in endospheres and 60 nodes in rhizospheres (Figure 6, Tables S3-S4 and S5-S6, respectively). ASVs in rhizospheres had a lower average node degree compared to endospheres (4.4 vs. 8.4; Figure6), indicating fewer strong associations between the microbiota in rhizospheres. Rhizospheres also had lower average edge weight values, suggesting more negative associations between taxa in their networks, compared to more positive associations among ASVs found in endospheres (0.274 vs. 0.341; Figure 6).

**DISCUSSION**

We investigated the patterns of microbial assembly in rhizospheres and root endospheres in natural populations of the non-model pan-palaeotropical C4 grass species, *Themeda triandra*. We found that rhizosphere and endosphere diversity patterns were consistent with the two-step selection process (Bulgarelli et al. 2013) – endospheres were less diverse than rhizospheres. We also observed convergence inendospheres across populations, where these bacterial communities were more homogeneous than rhizospheres. Despite this convergence, endosphere recruitment was also influenced by site-specific factors, including aridity. We found more unique bacterial ASVs in endospheres than rhizospheres, which suggests a potential role of vertical transmission (i.e., parent to offspring transfer) and/or life stage dependency on endosphere colonisation. Finally, we found that assembly processes in endospheres were more deterministic than neutral, and that there was a consistent core microbiome in these endospheres that probably supports the functioning of *T. triandra*. A deeper understanding of these microbial interactions would help inform soil resource management during conservation and restoration efforts (e.g., propagation, translocation, revegetation), especially for those that include *Themeda triandra*.

*Two step selection process*

We observed that overall bacterial diversity in *T. triandra* endospheres was approximately 48% lower than in rhizospheres. Both endospheres and rhizospheres had distinct community compositions, which is consistent with the two-step selection process. Our diversity and composition findings are in line with previous work on *Arabidopsis thaliana*, where several studies have now shown the selection of microbiota across soil and rhizosphere environments into root endospheres (Bulgarelli et al. 2013, Urbina et al. 2018, Barajas et al. 2020). These previous studies suggest that the controlled release of exudates by the plant attracts and supports the recruited microbiota (Bai et al. 2022). Though we did not directly measure root exudates, we do find compelling results from a bacterial community perspective and, together with our previous *T. triandra* soil-rhizosphere study (Hodgson et al. 2024), thus present strong evidence that the two-step selection process is active in this non-model, keystone grass species. Importantly, we report these results from naturally occurring populations of this non-model grass species which differs from previous studies, which generally focussed on plants growing *ex situ,* under controlled lab or greenhouse conditions.

Our detailed investigation into assembly patterns showed stronger deterministic processes in endospheres compared with rhizospheres, which again is consistent with the two-step processes – it is expected that the host plant has greater regulatory and selective control over microbiota inbound to roots than in rhizospheres (Bulgarelli et al. 2013). Interestingly we found major structural differences with several key phyla shifting across rhizospheres and endospheres. Furthermore, our endospheres contained more ASVs that deviated from neutral theory than rhizospheres, which suggests that the recruited ASVs may have important functions in *T. triandra* (Sloan et al. 2007, Stopnisek and Shade 2021). We note that while ASVs abundant in rhizospheres are primarily a result from the release of exudates by the host plant, the migration of these ASVs into the endosphere could be affected by microbe-microbe interactions, such as a priority effects – microbiota that colonise first may create conditions that allow other ASVs to arrive and thrive (Rillig et al. 2015, Debray et al. 2022). However, the fundamental structural differences suggest that there are important roles and traits of microbiota are selected for. Future work should characterise the root exudates involved and determine fitness consequences of these assembly processes.

*Endosphere convergence*

We report that local site conditions influenced endosphere recruitment dynamics, which resulted in a unique assortment of differentially abundant bacterial ASVs in the endospheres across sites – an effect also observed in previous studies on the trees *Populus deltoides* and *Taxodium distichum* (Gottel et al. 2011, Lumibao et al. 2020). Observing different endospheres across sites suggests that local conditions and/or resource availability affected how *T. triandra* regulates inbound microbiota (Vandenkoornhuyse et al. 2015). These influences were consistent with our earlier work which showed that *T. triandra* bulk soil microbial communities and rhizospheres were strongly shaped by soil nutrient levels, aridity and moisture availability (Hodgson et al. 2024). Local conditions are well known to shape bulk soil, rhizospheres and endospheres, however, in our study, these site-specific effects did not appear to impede the development of a convergent root endosphere across populations. Factors that shape internal microbial profiles could also shape preferential niches created by the host plant or some combination of other influences, such as microbe-mediated priority effects (Rillig et al. 2015). This raises intriguing questions about the functional potential of the ‘core’ microbial endosphere, and follow-up studies should investigate this further.

We reported higher overall complexity (based on node degree in our network analysis) and positive associations of ASVs in endospheres compared to rhizospheres, indicating remarkable symbiosis inherent in the convergence of bacterial communities in these root compartments. The top connected ASVs (= hub taxa) are often hypothesised to be keystone species that support or facilitate the recruitment of other microbiota (Rillig et al. 2015, Trivedi et al. 2020, Debray et al. 2022). Additionally, a decrease in the ratio of conditionally rare taxa to rare taxa within rhizospheres compared with endospheres (1.71 versus 3.84, respectively) shows that rhizospheres often support highly varied microbial community structures that are also more diverse (i.e., greater alpha diversity). As expected, we report stronger evidence of more symbiotic relationships in endospheres (i.e., less influenced by local soil and/or climatic conditions) relative to rhizospheres, which supported ASVs with fewer key microbe-microbe associations (Trivedi et al. 2020).

*Vertical transmission of microbiota*

The relatively high count of bacterial ASVs that were unique to root endospheres were likely populated via vertical transmission (i.e., from parent plant flowers to their offspring during seed development (Bulgarelli et al. 2013, Escobar Rodríguez et al. 2018, Abdelfattah et al. 2023); or transferred across host compartments (e.g., leaves or stems into roots (Chi et al. 2005)). Vertically transmitted bacterial endophytes are often involved in mobilising plant nutrients and affect phytohormone signalling inside roots (Bulgarelli et al. 2013, Santoyo 2022). Unique ASVs within each site could then be inherited through vertical transmission due to local adaptation of *T. triandra* populations (Thiergart et al. 2020, Durán et al. 2022). As such, this form of parent to offspring transfer could be critical for *T. triandra* fitness where microbiota cannot survive independently in soil environments, at least during part of their lifecycle, and host plants may evolve traits that facilitate the persistence of a portion of the microbial community (Johnston-Monje and Raizada 2011, Lumibao et al. 2020, Lyu et al. 2021).

It is worth considering that the ASVs suspected of vertical transmission in this study could still be a product of the two-step selection process, especially if we simply did not observe them in the rhizosphere during sequencing due to insufficient sequencing or the changing nature of rhizospheres through plant development.Further research could investigate how horizontally transferred bacterial taxa (i.e., soil to root endosphere colonisation) are supported in soil environments and whether they require their plant hosts for completion of their lifecycles (i.e., are they obligate symbionts?) (Vandenkoornhuyse et al. 2015). These ASVs may have a dormant, protected life stage (e.g., spore-forming) (van Vliet 2015), or could perhaps be microbiota that are influenced by host plant demographics and local adaptation (Ledeganck et al. 2003, Hannula et al. 2021). Further investigations should further investigate the vertical transmission of root endospheres.

*Conclusions*

We show that the microbiomes of natural populations of *T. triandra* growing across diverse environments retain assembly processes consistent with root endosphere colonisation from rhizospheres. We show that deterministic assembly processes acted strongly on these endospheres, as they were strongly affected by both environmental factors (e.g., aridity) plus host selection for a corerootmicrobiome. Additionally, while numerous endosphere taxa were likely from the plant rhizospheres, we present evidence for probable vertical transmission of microbiota from parent to offspring. Our limited understanding of the complex roles of plant-associated microbiota hinders our ability to harness the ecology of these important relationships in applied ecology context (e.g., propagation, translocation, revegetation). Future investigations should consider the functional roles and inheritance patterns of root endosphere microbiota in non-model plant species, and assess how these plant-microbe interactions effect host fitness.

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**DATA ACCESSIBILITY**: Data will be made publicly available on Figshare and Sequence Read Archive upon acceptance of this manuscript (Bioproject IDs: PRJNA1029310, PRJNA1138818).

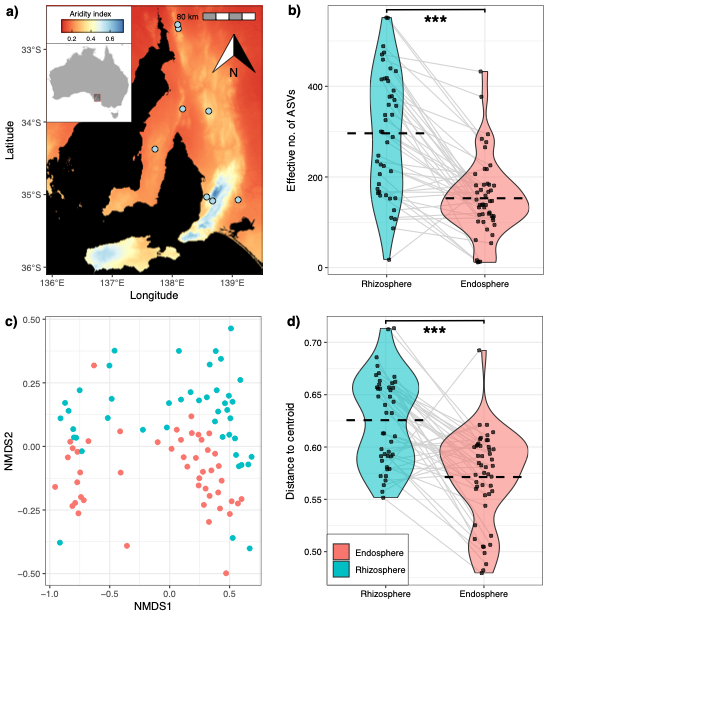
**BENEFITS GENERATED**: Benefits from this research accrue from the sharing of our data and results on public databases, as described above.

**AUTHOR CONTRIBUTIONS**: Project design: RJH, SR, MFB; Field work: RJH, CCD, MFB; Lab work: RJH, CCD; Statistical analysis: RH, CL; Writing manuscript: RH; Revisions and editing: RJH, CL, CCD, SR, MFB. All authors gave approvals before final submission.

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**AI USE STATEMENT**: We used artificial intelligence technology to assist in editing the manuscript (ChatGPT-3.5 by OpenAI). The AI was employed to identify potential areas of improvement. All AI suggestions were reviewed and revised in detail, and authors were responsible for implementing these revisions.

**FIGURES**



**Figure 1.** (a) Map showing Australia and the sampling locations of *Themeda triandra* populations (blue points) across a strong aridity gradient in southern Australia. (b) Bacterial alpha diversity as effective number of ASVs in *T. triandra* rhizospheres. (c) NMDS ordination showing the differences in bacterial community composition between rhizospheres (blue) and endospheres (red). (d) Distance to centroid of samples comparing rhizosphere (blue) and endosphere (red) samples, calculated from Bray-Curtis dissimilarity.

**Figure 2:** Bacterial ASV relative abundances visualised at the phylum level in endospheres and rhizospheres. (a) Stacked bars represent samples, grouped by aridity index of their sampling site. The bar colours represent the bacterial phylum. Chord diagrams for (b) rhizospheres and (c) endospheres, showing the relative proportion of each bacterial ASV within each phylum (groupings: A-K) found within bacterial abundance categories (AT – abundant taxa, MT – moderate taxa, RT – rare taxa, CAT – categorically abundant taxa, CRT – categorically rare taxa, and CRAT – categorically rare and abundant taxa). (d) Differential abundance analysis of major and minor bacterial phyla across the rhizospheres and endospheres.

**A diagram of different types of plants

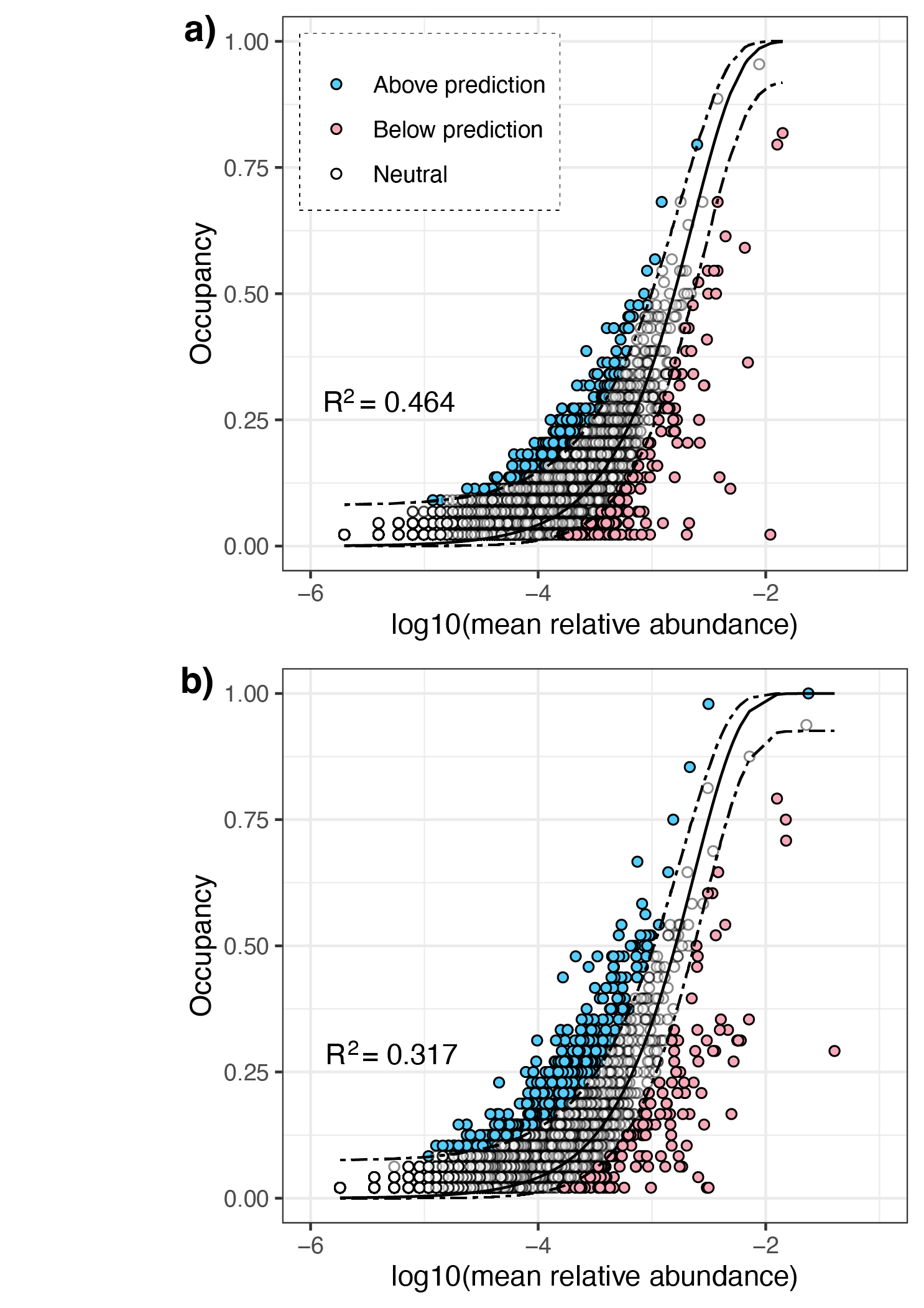
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**Figure 3.** (a) Venn diagram of unique ASVs across *T. triandra* endospheres and rhizospheres showing number of unique ASVs and percentage of reads within each grouping, and (b) plot summarising relative abundance of phyla for the unique and shared ASVs in the endospheres and rhizospheres. (c) Partial Venn diagram showing unique *T. triandra* rhizospheres ASVs across each sampling site, and shared across all sites, and percentage of reads within each grouping; and (d) partial Venn diagram of unique ASV across *T. triandra* endospheres in each site, and shared across all sites, showing number of unique ASVs and percentage of reads within each grouping.

A close-up of a graph

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**Figure 4.** (a) Heatmap showing 218 differentially abundant ASVs across *T. triandra* rhizospheres and endospheres and (b) the number of differentially abundant ASVs calculated within each sampling site. The negative grouping includes those ASVs favoured in endospheres (negative log fold change), whereas the positive grouping includes ASVs favoured in rhizospheres (positive log fold change). Sites are ordered from most to least arid (top to bottom, respectively). (c) Upset plot showing the number of shared and unique bacterial ASVs across each site. This plot shows the first 30 most populated ASV intersections between sites (see Figure S10 for all site intersections).

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**Figure 5.** Abundance-occupancy curves fitted with the Sloan neutral modelin *T. triandra* (a) rhizospheres and (b) endospheres. Each point represents a bacterial ASV that occurs above (blue), below (pink) , or within (white) neutral model predictions. ASVs that occur at greater occupancies than predicted by the neutral model (blue) are hypothesised to be positively selected by the environment, and those occurring with lower occupancies than predicted by the neutral model (pink) are hypothesised to be negatively selected by the environment.

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**Figure 6.** Network analysis of bacterial ASVs in (a) rhizospheres and (b) endospheres, sampled across *T. triandra* aridity gradient. Vertex colour indicates taxonomic groups at the ASV level. Positive associations are represented by turquoise edges, and negative associations are represented by red edges. The average degree and average edge weight is shown below of each network.

**TABLES**

**Table 1.** *T. triandra* sampling sites across southern Australia.

|  |  |  |  |
| --- | --- | --- | --- |
| Site name | Latitude, longitude | Aridity index | Sampling date |
| Alligator Gorge | -32.71487, 138.10172 | 0.445 | 15 Dec 2021 |
| Barlunga Gap | -33.82000, 138.17392 | 0.347 | 14 Dec 2021 |
| Frahn’s Farm | -35.07231, 139.09781 | 0.454 | 19 Dec 2021 |
| Maitland | -34.37366, 137.71203 | 0.453 | 21 Dec 2021 |
| Mount Maria | -32.65862, 138.08985 | 0.318 | 16 Dec 2021 |
| Neagles Rock Reserve | -33.85031, 138.60674 | 0.651 | 14 Dec 2021 |
| Scott Creek | -35.08720, 138.67266 | 0.903 | 19 Dec 2021 |
| Sturt Gorge | -35.03311, 138.57324 | 0.634 | 13 Dec 2021 |

**Table 2.** Bacterial ASVs allocated to six relative abundance categories.

|  |  |  |  |
| --- | --- | --- | --- |
| Compartment | Category | Number of ASVs | Number of sequences |
| *Rhizosphere* ~ | Abundant taxa (AT) | 0 | 0 |
|  | Moderate taxa (MT) | 11 (0.15%) | 7821 (1.55%) |
|  | Rare taxa (RT) | 1477 (20.45%) | 5518 (1.09%) |
|  | Conditionally abundant taxa (CAT) | 4 (0.06%) | 19238 (3.81%) |
|  | Conditionally rare taxa (CRT) | 5705 (78.99%) | 429588 (84.97%) |
|  | Conditionally rare and abundant taxa (CRAT) | 25 (0.35%) | 43439 (8.59%) |
| *Endosphere* ~ | Abundant taxa (AT) | 1 (0.01%) | 13094 (2.37%) |
|  | Moderate taxa (MT) | 6 (0.07%) | 6755 (1.23%) |
|  | Rare taxa (RT) | 2970 (36.69%) | 11938 (2.16%) |
|  | Conditionally abundant taxa (CAT) | 4 (0.05%) | 31755 (5.76%) |
|  | Conditionally rare taxa (CRT) | 5070 (62.63%) | 322711 (58.51%) |
|  | Conditionally rare and abundant taxa (CRAT) | 44 (0.54%) | 165315 (29.97%) |