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Microbial communities associated with distance- and density-dependent seedling mortality in a tropical rainforest

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Abstract The high levels of diversity within tropical rainforest communities has been linked to non-random patterns of seedling mortality with several studies implicating pathogenic plant–microbe interactions in driving mortality processes. Despite the proposed importance of microorganisms in maintaining rainforest diversity, few studies have investigated soil community dynamics in relation to non-random mortality processes. A mechanistic understanding of microbial processes that help create rainforest diversity is critical for the conservation of these ecosystems. This study investigated microbial community dynamics that may underpin distance- and density-dependent mortality in the long-term forest dynamics plot, Davies Creek, in tropical Far North Queensland using community fingerprinting. We hypothesized that: (1) microbial involvement in distance-dependent seedling mortality would result in an increase in community similarity or the presence of predictor OTUs in conspecific adult tree rhizospheres, relative to physically nearby heterospecifics; (2) on average, plant species identified as having a history of distance dependent seedling mortality would exhibit more similar microbial communities among their conspecific individuals, than those that did not; and (3) dense patches of conspecific seedlings would

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promote the assembly of distinct soil microbial communities, which may be involved in density-dependent seedling mortality. We found no evidence of rhizosphere community similarity amongst adult plant rhizospheres. However, the presence of densely germinating seedlings altered the soil communities relative to seedling-sparse soils, enriching different OTUs depending on the patch location.

Keywords Janzen–Connell · Rainforest diversity · Soil pathogens · Tropical

Introduction

Tropical rainforests are amongst the most complex and diverse ecosystems on the planet (Novotny et al. 2006; Wilson et al. 2012). In less than 1 km^2, rainforests can harbour three times the plant diversity of entire temperate forests (Wright 2002). The immense plant and animal diversity that rainforests harbour provide economic benefits through eco-tourism (Prideaux 2014) and continue to contribute to the discovery of new therapeutics (Balunas and Kinghorn 2005; Perigo et al. 2016) and novel species (Jay et al. 2016; McDonald et al. 2016). The importance of conserving these biodiversity hotspots is without question, yet the mechanisms that maintain rainforest diversity are yet to be fully understood (Wright 2002; Terborgh 2012). This understanding will be critical for protecting and conserving rainforests in the face of a changing climate.

A role for soil microbial communities in maintaining rainforest diversity has been hypothesized almost as long as rainforest diversity maintenance has been a subject of ecological interest (Janzen 1970; Connell et al. 1984; Wright 2002; Mangan et al. 2010; Bever et al. 2015). By altering the composition of seedling communities via distance- or density-dependent seedling mortality, microbial phytopathogens have the potential to 'engineer' entire mature rainforest communities (Comita et al. 2014). Distance-dependent seedling mortality is hypothesized to be a consequence of host-specific pathogen reservoirs, maintained in the adult rhizosphere, which increase the *per capita* probability of seedling mortality near conspecific adults. Similarly, density-dependent seedling mortality is thought to occur due to high-conspecific seedling density attracting and sustaining high pathogen loads (Augspurger and Kelly 1984). Because common species have a higher probability of geminating near a conspecific adult, or other seedlings, both mechanisms of non-random mortality promote diversity by thinning out common species whilst allowing rare species to grow unimpeded.

Despite the proposed importance of microorganisms in the maintenance of tropical macro-community diversity, few microbial-centric attempts have been made to understand the ecology of the soil communities that drive seedling mortality. To date, the body of evidence that implicates soil pathogens in non-random mortality processes has come primarily from studies manipulating seedlings in situ (Augspurger 1983, 1984; Augspurger and Kelly 1984; Pringle et al. 2007; Mangan et al. 2010) or studies that employ soil fumigation (Bell et al. 2006; Norghauer et al. 2010; Bagchi et al. 2014; Liu et al. 2015). Some studies of microbial involvement in non-random mortality have focused on specific subsets of the soil community such as known pathogenic microorganisms (Gilbert et al. 1994) or the beneficial partners that can protect against pathogen attack (Bachelot et al. 2017). These studies utilize a priori knowledge of pathogenic/beneficial plant–microbe interactions. This approach is limiting as phytopathogens and beneficial microbes cannot be ascribed to a discrete set of taxonomic identifiers, and genera that contain multiple well-known pathogens also contain commensal and beneficial microorganisms (Rodriguez et al. 2008; Zhang et al. 2012; Aoki et al. 2014; Barka et al. 2016). Additionally, the role of 'pathogen' cannot be unconditionally ascribed to an individual species because pathogenicity is conditional upon abiotic and biotic interactions (Da Silva et al. 2002; Smith and Reynolds 2015).

Studies of non-random seedling mortality that inspect the dynamics of associated microbial communities are lacking. Understanding non-random mortality processes using a holistic microbial community approach avoids the need for a priori knowledge of pathogenic or beneficial plant–microbe interactions and facilitates the study of multiple pathogen interactions in non-random mortality as well as the involvement of community members that are less-intensively investigated, such as the bacterial component which is not affected by fumigation techniques (Lamichhane et al. 2017). Moreover, a deeper understanding of the soil community dynamics in tropical ecosystems

could assist in the identification of microbiological indicators of ecosystem resilience (Chaer et al. 2009; Griffiths and Philippot 2013) that could be used in the conservation and monitoring of rainforests, which are predicted to be impacted by climatic change in the next 50 years (Hilbert et al. 2001; Hughes 2003; Pecl et al. 2017).

We report a community-level approach to investigating microbial driven distance- and density-dependent mortality in a tropical ecosystem using automated ribosomal intergenic spacer analysis (ARISA) (Fisher and Triplett 1999). A survey of seedling mortality patterns, that incorporated over 50-years of plant demographic data from the Davies Creek study site, in far-north Queensland, Australia, was used to test the hypothesis that distance-dependent seedling mortality was present for particular tree species. This survey was made possible by the Davies Creek data set which is remarkable for its inclusion of the smallest tree size classes, including seedlings, and was used to inform the microbial community experimental design (Connell et al. 1984; Connell and Green 2000; Green et al. 2014).

Distance-dependent mortality was investigated by examining the rhizosphere microbial community dynamics of 100 adult trees, constituting 10 focal species with known patterns of distance-dependent mortality. We hypothesized that: (1) microbial involvement in distance-dependent seedling mortality would be revealed as an increase in community similarity or the presence of predictor operational taxonomic units (OTUs) in conspecific rhizospheres, relative to spatially nearby heterospecific rhizospheres; and (2) rhizosphere communities would be more similar among the conspecifics of tree species documented to exhibit distance-dependent seedling mortality at Davies Creek, compared to those that do not. Density-dependent mortality was examined at local scales, by comparing soils associated with dense seedling patches of a representative species of rainforest tree to adjacent seedling-sparse soils. We hypothesized that dense patches of conspecific seedlings would promote the assemblage of distinct microbial communities, which may be involved in density-dependent seedling mortality.

Methods

Davies Creek study site

This experiment was conducted in primary tropical rainforest occurring on comparatively low-fertility soils derived from granite bedrock at 800–900 m elevation in the Lamb Range of northern Queensland, Australia. The study site is described fully in Connell et al. (1984). The 1.7-ha site has never been logged and is surrounded by continuous rainforest that is fully protected under World Heritage status. The site receives 3000 mm of rain annually, with most of this falling December–April. All trees $\geq$ 10 cm at breast height (dbh) were tagged, mapped and measured for girth in 1963. The site contains a total of 120 species with stem diameters $\geq$ 10 cm dbh encompassing a basal area of 61 m^2/ha (Connell et al. 2005). Recruitment into this size class, and growth and mortality have been tracked at regular intervals. In 1965, individuals in smaller size classes, down to newly germinated seedlings, were mapped along transects through the plot, and seedling recruitment and mortality have been tracked at regular intervals since (see Connell et al. (1984), Connell and Green (2000) and Green et al. (2014) for full details and schedules of recruitment and mortality censuses).

Selection of focal tree species using long-term demographic data

Long-term tree demographic data from the Davies Creek site were used to identify tree species that exhibit evidence of distance-dependent seedling mortality, and to select the final species list for soil microbial sampling via additional criteria.

To determine if the probability of seedling mortality was dependent on nearest neighbour distance, a custom R script (R Core Team 2018) was created to calculate the distance between each seedling recruit and its nearest conspecific large tree (individuals $\geq$ 10 cm dbh) for use in logistic regressions. In these analyses “seedling recruits” were those plants germinating on the permanent transects after the initial 1965 survey, and between 15 seedling recruitment surveys up to and including 2003. Seedlings that recruited after 2003 were not included because mortality is best characterised over longer than shorter periods of time. Restricting seedling recruits up to and

including 2003 ensured there were at least 10 years (mortality surveys in 2006 and 2013) by which to determine each seedling's long-term fate.

The fates of many seedlings were assessed over longer periods according to how long before 2003 (and after 1965) they recruited. The population of conspecific large trees under which seedlings recruit changes over time because some trees die, and others recruit into that size class. As such the potential nearest large conspecific tree, for each seedling recruit, was restricted to those individuals that were $\geq$ 10 cm dbh in the year when the seedling recruited and still alive until at least the next mortality survey.

The final list of focal species that could potentially be targeted for field sampling of microbial communities was restricted to species that had records for at least 100 seedling recruits that could be used for analyses of distant-dependent mortality, and that had at least ten large trees alive on the plot for sampling in 2014. This restricted the list of potential species on the site to 21, from which 100 trees representing 10 focal species were chosen such that six species exhibited density-dependent seedling mortality and four species did not (Table 1). In the absence of information about the extent of the possible zone of influence of focal trees on soil microbiota (via leaf litter, rain throughfall or roots), we conservatively estimated this zone could extend as far as two canopy-radii from the trunk, and that for sample independence only conspecific trees with non-overlapping zones of influence could be considered. We used cross-species allometric equations developed for tropical trees elsewhere (Poorter et al. 2006) to estimate canopy radius from dbh, and then plotted on maps the zone of influence around focal large trees. The maps were inspected to choose ten trees in each focal species whose zones of influence did not overlap. Although these estimated zones of influence were necessarily rough estimates, they were only designed as an aid for tree selection and most of the sampled conspecific large trees were many canopy radii distant from each other.

Sampling soil microbial communities

Soil communities closely associated with adult trees (ten trees for each of the ten selected focal species)

Table 1 Tree species investigated in this study and their observed mortality pattern

Family	Species	Number of seedling recruits 1967–2003	Number surviving to 2013	χ^2	Odds ratio	Distance dependence mortality
Ochnaceae	*Brackenridgea nitida*	138	24	0.11	1.036	PDD
Proteaceae	*Cardwellia sublimis*	828	14	0.06	1.004	None
Cunoniaceae	*Ceratopetalum succirubrum*	6164	414	96.39***	0.949	NDD
Lauraceae	*Cryptocarya angulata*	1576	48	9.79**	0.982	NDD
Lauraceae	*Endiandra wolfei*	175	42	0.29	1.000	None
Malvaceae	*Franciscodendron laurifolium*	1260	50	5.76*	1.041	PDD
Clusiaceae	*Garcinia* sp. nov	350	18	0.20	1.006	None
Sapotaceae	*Niemeyera* sp. nov	14,056	1106	11.87***	0.983	NDD
Euphorbiaceae	*Rockinghamia angustifolia*	712	5	4.09*	0.935	NDD
Myrtaceae	*Syzygium endophloium*	756	18	0.02	0.999	None

χ^2 is the test statistic from the logistic regression analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), Odds ratio from the logistic regression. For distance-dependent mortality: *PDD* positive distance dependence (probability of seedling mortality increases with distance from conspecific large tree), *NDD* negative distance dependence (probability of seedling mortality decreases with distance from conspecific large tree), none = probability of mortality unrelated to distance

were sampled by taking five replicate soil cores (approximately 2.5 cm diameter to 10 cm depth) 1 m out from the base of each tree at 60°, 110°, 180°, 270°, and 360° from magnetic North. Soil samples were refrigerated until DNA was extracted. To disentangle abiotic and biotic influences upon the soil community, soil moisture, pH, EC and elevation were determined. 5 g of each soil sample was used to determine soil moisture by oven drying at 105 °C for 24 h. After air drying, replicate samples were bulked and used to determine pH (1:5 0.01 M $CaCl_2$ method) and electrical conductivity (EC; 1:5 water method). Elevation associated with each sample was determined from contour maps and absolute X and Y coordinates of each tree.

Sampling soil microbial communities associated with dense assemblages of recently germinated seedlings was necessarily opportunistic, requiring mass germination events to coincide with re-censusing expeditions. As such, data for only one species, *Cryptocarya angulata,* were obtained. Soils associated with recently germinated (< 18 months old) dense patches of *C. angulata* seedlings were sampled in May 2015 at three locations on the plot. At each seedling patch, soil cores were collected from under the zone of densely germinating seedlings (minimum eight replicates; soil did not include seedling rhizospheres) and from areas on either side of the seedling zone (minimum four replicates each side) along a contour line (Fig. S2). Soil samples were refrigerated until DNA was extracted. The sampling of seedling sparse zones from either side of the dense seedling zone was done to ensure that any clustering between communities from seedling-dense and seedling-sparse soils could not be attributed to spatial variation.

gDNA extraction and community profiling of soil communities

Prior to gDNA extraction, the five replicate soil cores from each of the adult trees were bulked and mixed thoroughly. Replicates from seedling patches were kept separate. Community gDNA was extracted from soils (0.25 g) using a MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories Inc; CA, USA) as per manufacturer's instructions. DNA concentrations were recorded using an Implen P330 Nanophotometer (Implen GmbH; Munich, Germany). All samples were normalized to working concentrations of 5 ng/µl and stored at – 20 °C until required.

Automated ribosomal intergenic spacer analysis (ARISA) is a community fingerprinting technique that uses non-coding DNA fragments between conserved rRNA genes to measure microbial community structure and diversity. Operational taxonomic units (OTUs) are assigned within communities based on amplicon length polymorphisms and OTU abundances are estimated via the relative fluorescence intensity associated with each OTU. ARISA-PCR amplifications were carried out in 20 µl reactions using a TProfessional TRIO combi-thermocycler (Biometra GmbH; Göttingen, Germany). Reaction mixtures contained: 2.5 U TopTaq DNA polymerase (Qiagen); 0.5 pM of dNTP mix; 0.5 pM of each appropriate primer; 3 pM of $MgCl_2$ and 10 ng of community DNA.

Bacterial ARISA-PCR used primer pairs *16S-1392F* and *23S-125R* (Anderson and Cairney 2004; Kovacs et al. 2010). Cycle settings used for bacterial PCR reactions were as follows: initial denaturation, 3 min at 94 °C; 33 cycles of 1 min denaturation at 94 °C; 1 min annealing at 52 °C; 1.5 min extension at 72 °C; and 6 min final extension at 72 °C (Kovacs et al. 2010). Fungal ARISA-PCR used primer pairs *ITS1F* and *ITS4* (Anderson and Cairney 2004). Cycle settings used for fungal PCR reactions were: initial denaturation, 2 min at 96 °C; 30 cycles of 1 min denaturation at 96 °C; 1 min annealing at 55 °C; 2 min extension at 72 °C; and 10 min final extension at 72 °C (Lee and Taylor 1992). For oomycete assemblages, primer pairs *ITS4-ITS6* and ITS6-ITS7 were used for successive rounds of PCR amplification in a semi-nested approach to minimise fungal ITS amplification, as described in Sapkota and Nicolaisen (2015). The thermal cycle settings for the first oomycete PCR were: denaturation, 5 min at 94 °C; 17 cycles of 30 s denaturation at 94 °C; 30 s annealing at 55 °C; 1 min extension at 72 °C; and 10-min final extension at 72 °C. The second PCR was identical to the first except that the annealing temperature was increased to 59 °C and 35 cycles were conducted. For each PCR reaction, 10 µl of unpurified PCR product was submitted to the Australian Genome Research Facility (AGRF; Melbourne, Australia) for fragment separation analysis via capillary electrophoresis on an Applied Biosystems 3730 DNA Analyser (Life Technologies; CA, USA). The data

were analysed using GeneMapper software (Applied Biosystems; CA, USA).

Generation of ARISA OTU tables

Raw ARISA data (fragment size and peak area) were obtained from AGRF. A minimum cut-off of 50 fluorescence units was used to exclude background noise. Sample × OTU matrices containing binned OTUs were produced in R version 3.1.1 (R Core Team 2018 using the 'automatic' and 'interactive' binning scripts created by Ramette (2009) in the R programming language. Bin sizes of 2, 3.5 and 3.5 bp were determined to be optimal for bacterial, fungal and oomycete datasets, respectively. Under sequenced communities were defined as those with OTUs < 10% of mean number of OTUs and were removed from the dataset: 13 bacterial samples were removed. Singletons were removed to prevent sporadically detected OTUs from influencing community analyses. Although ARISA datasets are normalized to relative abundance via the algorithms used in the binning strategy, fungal and oomycete count data were further log(x + 1) transformed to down-weight the influence of over-dominant OTUs. After transformation, one fungal assemblage (Tree_ID 177) was identified with an over abundant OTU and removed from the data.

Statistical analysis

All the statistical analyses were conducted in the R environment, version 3.2.2 (R Development Core Team 2018). To explore the relationships between environmental variables (pH, EC, soil moisture, elevation and plant species ID) and community composition, environmental variables were z-transformed and a canonical correspondence analysis (CCA) was conducted using the *cca* function in *vegan* (Jari Oksanen 2019). The *ordistep* function with both forward and reverse procedures selected the best subsets of environmental variables with a significant correlation to community data. This procedure implements a permutation test (999 permutations) to select variables that significantly improve the model fit. The *anova.cca* function was used to confirm the significance of variance explained by the whole model and each explanatory variable. Variance Inflation Factors (VIFs) were used to confirm selected variables did not exhibit collinearity. The VIF any given variable was < 2.

Permutational MANOVAs (PERMANOVAs) of Bray–Curtis dissimilarity scores were used to determine whether conspecific rhizosphere communities were more similar compared to nearest neighbour rhizospheres via the *adonis* function. pH was determined to be a significant abiotic driver of community structure at the plot level and was added as a secondary factor to PEMANOVA analyses. Indicator species analysis was conducted using the *indval* function in *labdsv* to identify OTUs that were associated with the rhizospheres of different adult tree species (Dufrêne and Legendre 1997).

To investigate patterns of community dissimilarity amongst seedling patches, the Bray–Curtis coefficient was used in conjunction with non-metric multidimensional scaling (nMDS). Analysis of similarities (ANOSIM) with 999 permutations was used to test for an effect of seedling density upon soil communities via the function *anosim* (Clarke 1993; Anderson 2001; Jari Oksanen 2019).

Results

Elevation is a major driver of community structure at the plot scale

Variable selection based on the *ordistep* function identified elevation as a major environmental driver of bacterial, fungal and oomycete assemblage composition at the plot scale, with pH and EC contributing to bacterial community structure and pH and soil moisture contributing to oomycete assemblage structure (Fig. 1). In all cases pH (range 3.3–4.7) negatively correlated with plot topography measured as elevation. Constraining variables collectively explained 7.5, 2.6 and 8.2% of bacterial, fungal and oomycete assemblage variation, respectively.

Community fingerprinting revealed little evidence of increased rhizosphere community similarity amongst conspecific adult trees

We hypothesized that the presence of species-specific pathogen reservoirs in adult tree rhizospheres, which could facilitate distance-dependent seedling mortality, would present as an increase in similarity amongst

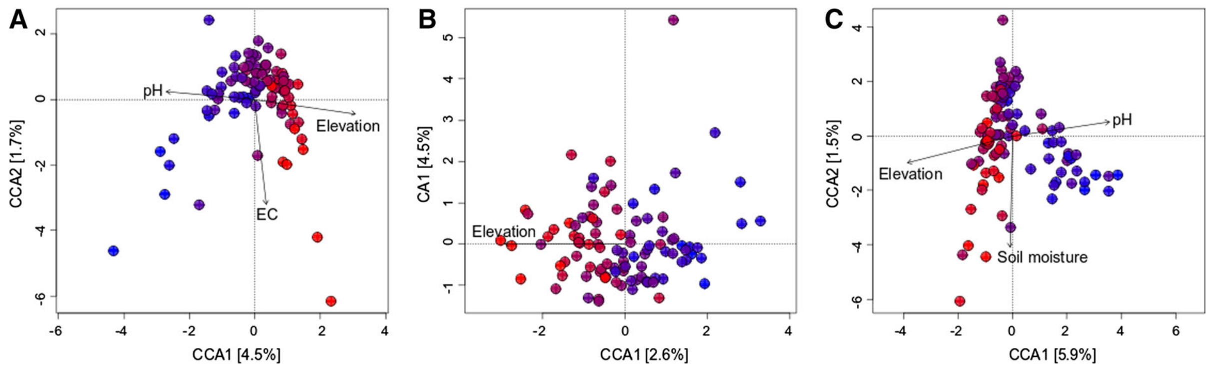

Fig. 1 CCA of bacterial (**a**), fungal (**b**) and oomycete (**c**) assemblages. Vectors represent physicochemical parameters that significantly impact community structure. EC = Electro conductivity. Rhizosphere communities are coloured by elevation with warmer colours indicating higher elevation

rhizospheres from conspecific trees compared to rhizospheres from spatially nearby heterospecific trees. To account for the observed impact of elevation on community structure, Elevation was incorporated into the PERMANOVA model. However, we found no evidence of species-specific bacterial or oomycete rhizosphere assemblages using PERMANOVA (Table 2). Two tree species, *Garcinia* sp. nov. and *Cardwellia sublimis* had species-specific fungal assemblages there were significantly different from assemblages associated with nearest neighbour trees (Table 2). However, neither tree species has a documented history of distance-dependent mortality at Davies Creek (Table 1).

We used indicator species analysis to identify the presence of individual OTUs associated with each given focal species. 26 indicator OTUs were associated with eight of the ten tree species. 12 indicator OTUs were associated with *Garcinia* sp. nov. which has no documented history of distance-dependent mortality at Davies Creek. In general, there was no pattern regarding tree species with a history of distance-dependent mortality and the presence of indicator OTUs. Indicator OTUs generally represented rare community members (< 1% relative abundance), one oomycete OTU (OTU_233.9) had a relative abundance of 6.3%.

Seedling density altered soil microbial community structure

Within the three *C. angulata* seedling patches that were identified at the time of sampling, multiple seedlings were observed to be in a diseased state (Fig. S2). As such, we hypothesized that dense patches of conspecific seedlings would promote the assembly of distinct microbial communities which may be involved in density-dependent seedling mortality. Soils were sampled from dense patches of germinating *C. angulata* and adjacent seedling-sparse soils at three distinct locations on the Davies Creek plot. Location was a significant main effect for all ARISA-community profiles, however, seedling density significantly altered the structure of bacterial assemblages and there was a significant interaction between location and seedling density for oomycete assemblages (Table 3). Within each seedling patch, bacterial assemblages were significantly different between seedling-dense and seedling-sparse soils at two of the three sampling locations (Fig. 3, Table 4).

Discussion

Understanding soil microbial dynamics associated with non-random mortality patterns is essential for developing a mechanistic understanding of diversity maintenance in tropical ecosystems and the identification of microbiological indicators of rainforest soil health. At our study site, Davies Creek, the entire microbial community was significantly influenced by

Table 2 F-statistics and significance values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) for PERMANOVA testing the effect of tree species and pH on the structure of bacterial, fungal and oomycete ARISA profiles using Bray–Curtis distances

Tree species	Species-specific effect		pH effect		Interaction		Res
	Pseudo-F	d.f	Pseudo-F	d.f	Pseudo-F	d.f	d.f
Bacterial data							
B. nitida	0.726	1	2.836***	1	1.055	1	12
C. sublimis	0.913	1	2.421**	1	0.834	1	10
C. succirubrum	0.649	1	3.777***	1	0.784	1	15
C. angulata	0.869	1	2.569***	1	1.091	1	12
E. wolfei	0.754	1	1.443	1	0.609	1	15
F.laurifolium	0.908	1	3.635***	1	0.953	1	15
Garcinia sp. nov	0.957	1	2.695***	1	1.088	1	13
Niemeyera sp. nov	0.853	1	4.518***	1	0.726	1	12
R. angustifolia	0.651	1	2.959**	1	0.714	1	14
S. endophloium	0.750	1	2.977***	1	0.806	1	13
Fungal data							
B. nitida	0.988	1	1.460	1	1.044	1	16
C. sublimis	1.696*	1	2.117***	1	1.010	1	15
C. succirubrum	0.841	1	2.745***	1	0.898	1	16
C. angulata	1.325	1	1.471	1	0.859	1	16
E. wolfei	1.060	1	1.712	1	0.821	1	16
F.laurifolium	0.727	1	2.865**	1	0.853	1	16
Garcinia sp. nov	1.621*	1	1.829*	1	1.606*	1	15
Niemeyera sp. nov	0.684	1	1.464	1	0.661	1	15
R. angustifolia	0.639	1	1.393	1	0.690	1	16
S. endophloium	1.259	1	1.881	1	0.932	1	15
Oomycete data							
B. nitida	0.507	1	2.449*	1	0.307	1	16
C. sublimis	0.768	1	4.744**	1	1.395	1	16
C. succirubrum	1.146	1	4.199***	1	0.934	1	16
C. angulata	1.086	1	2.732**	1	1.463	1	16
E. wolfei	1.253	1	2.035*	1	0.775	1	16
F.laurifolium	0.856	1	2.289*	1	0.417	1	16
Garcinia sp. nov	1.373	1	1.835*	1	1.535	1	16
Niemeyera sp. nov	1.305	1	4.038***	1	0.997	1	16
R. angustifolia	0.727	1	4.127***	1	0.703	1	16
S. endophloium	0.947	1	1.532	1	1.262	1	16

Table 3 F-statistic and significance values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) for PERMANOVA testing the effect of sample location and seedling density on the structure of bacterial, fungal and oomycete ARISA assemblage profiles using Bray–Curtis distances

Data type	Location effect	Seedling density effect	Interaction
Bray–Curtis distances			
Bacterial-ARISA	$0.34_{(2,52)}$**	$0.03_{(1,52)}$**	$0.04_{(2,52)}$*
Fungal-ARISA	$0.15_{(2,52)}$**	$0.01_{(1,52)}$	$0.04_{(2,52)}$
Oomycete-ARISA	$0.24_{(2,52)}$**	$0.01_{(1,52)}$	$0.05_{(2,52)}$*

Table 4 R values for ANOSIM comparing soils under dense conspecific seedling patches to soil communities outside of the seedling patches (*$p < 0.05$)

Community type	Patch 1	Patch 2	Patch 3
Bray–Curtis distances			
Bacterial-ARISA	0.15*	− 0.05	0.32*
Fungal-ARISA	− 0.04	− 0.04	0.09
Oomycete-ARISA	0.03	− 0.03	0.11

elevation and bacterial-, and oomycete-assemblages were both influenced by pH, which was negatively correlated with elevation (Pearson's r = − 0.657; Fig. 1, Table 2). The importance of pH as a driver of bacterial assemblage has been reported independently numerous times at various spatial scales and across multiple soil types (Nuccio et al. 2016; Tripathi et al. 2016; Chen et al. 2019). Similarly, the negative correlation between elevation and pH has been reported in tropical research previously and is attributed to slope processes gradually leeching dissolved minerals such as exchangeable Ca and Mg (Chen et al. 1997; Tsui et al. 2004). Collectively, environmental drivers of soil community assembly only accounted for 2.6–8.2% of community variation. The low level of variation explained by environmental variables in this study suggests that additional drivers influence soil microbial community structure such as soil resource or light availability which have been shown to drive tropical plant community assembly (Baldeck et al. (2012); McCarthy-Neumann and Nez 2013).

Surveys of distance-dependent mortality at Davies Creek identified 10 tree species, 6 of which exhibit non-random morality patterns, that were suitable for further analysis of the associated soil microbial community. After accounting for the influence of elevation, microbial community fingerprints generally did not support our hypothesis that conspecific adult trees would exhibit similarities in their rhizosphere communities due to the presence of species-specific pathogen reservoirs (Table 2, Fig. 2). This was true for both community-level analyses, which accounted for abiotic drivers of community structure, and indicator OTU analysis. Only two tree species (*C. sublimis* and *Garcinia* sp. nov) were identified as having fungal assemblages which were more similar amongst

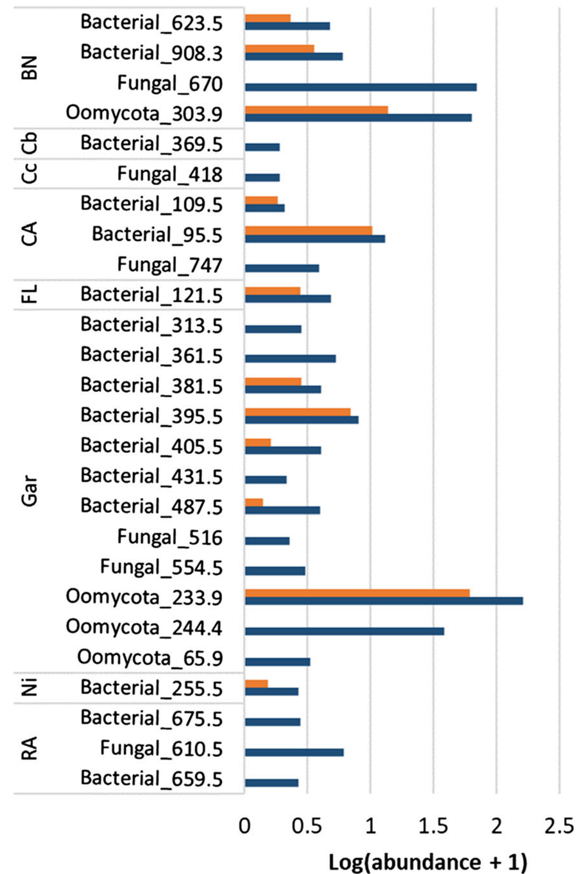

Fig. 2 Indicator species analysis comparing adult rhizosphere communities to nearest neighbour rhizospheres. For each indicator OTU, the log(OTU abundance + 1) in the rhizospheres of each tree species (blue) and in their associated nearest neighbour rhizospheres (orange) is displayed. Indicator OTUs are grouped by the tree species they were associated with, and are identified as belonging to bacterial, fungal or oomycete communities. BN = *B. nitida*, Cb = *C. sublimis*, Cc = *C. succirubrum*, CA = *C. angulata*, FL = *F.laurifolium*, Gar = *Garcinia* sp. nov., Ni = *Niemeyera* sp. nov., RA = *R. angustifolia*

conspecifics (Table 2) and *Garcinia* sp. nov. was the only tree species to be associated with a notable number of indicator OTUs. However, neither *C. sublimis* nor *Garcinia* sp. nov have a history of distance-dependent seedling mortality at Davies Creek. Possibly, the similarity in fungal assemblages reflect plant–fungal interactions that do not contribute to seedling mortality patterns, such as endo- or ectomycorrhizal associations. Additionally, species belonging to the genus *Garcinia* include important medicinal species which contain bioactive compounds including xanthones, biflavonoids and benzophenones (Iinuma et al.

1994; Maňourová et al. 2019). Xanthones from *Garcinia* species, of which over 50 have been identified in *G. mangosteen* alone, display a wide range of activities amongst which antimicrobial activity features prominently (Mackeen et al. 2000; Pedraza-Chaverri et al. 2008; Kuete 2010). As such, the abundance of *Garcinia* predictor OTUs may reflect the presence of bioactive compounds in the rhizosphere which shape the soil community.

It is possible that the non-random mortality patterns detected in our survey of plant demographic data were driven by vectors other than soil microbiota. While the possibility of insect-driven seedling mortality has not been tested at this study site, the hypothesis that vertebrate seeding predators drive non-random seedling mortality has been tested previously and only one of our focal species (*Niemeria* sp. previously *Chrysophyllum* sp.) exhibited evidence of vertebrate driven negative density dependence (Theimer et al. 2011). Whilst we found no evidence to support the presence of species-specific pathogen reservoirs, both shade house and field experiments have revealed evidence of negative plant-soil feedbacks which most certainly indicate that the host-specificity of soil pathogens has a role to play in shaping rainforest diversity (Augspurger 1984; Mangan et al. 2010; Eck et al. 2019). It is possible that our strategy of collecting rhizosphere samples 1 m from the base of adult trees precluded the collection of active rhizosphere communities, which are concentrated at growing root-tips. This sampling strategy was chosen to minimize interference from overlapping rhizosphere communities, and our demographic data indicated that seedlings of species that exhibit distance-dependent mortality germinate within 1 m of the adult stem (Table S1). Previous research using a similar sampling strategy was able to detect plant–microbe associations with the non-arbuscular mycorrhizal fungal community (Schappe et al. 2017). More likely, species-specific pathogen reservoirs (if they were present) are maintained at low abundances and were below the detection limit of ARISA, which is typically restricted to the most abundant community members (Gobet et al. 2014). It is also possible that the detection of unique pathogen reservoirs requires resolution of plant genotypes, not just species (Eck et al. 2019). As such, future work using next-generation sequencing may yet uncover evidence of species-specific soil communities with the potential to facilitate distance-dependent seedling mortality at Davies Creek.

Community fingerprinting data did support our hypothesis that dense patches of conspecific seedlings would promote the assembly of distinct microbial communities. As was seen in the adult tree rhizospheres, there was a significant impact of sample location on soil communities associated with patches of germinating *C. angulata* seedlings and communities from the three seedling patches were structurally distinct (Table 4, Fig. S3). Despite the high beta-diversity, a significant impact of conspecific seedling density upon bacterial assemblages was detected at two of the three patches tested (Fig. 3, Table 4).

It is well documented that plant root exudates alter rhizosphere microbial community structure and ecology (Bertin et al. 2003; Berg and Smalla 2009; Wood et al. 2018). It is possible that the observed changes to bacterial community structure reflect a microbial response to carbohydrate input which, due to the density of the germinating seedlings, was able to be detected beyond the typical zone of root influence of 1–2 mm. Our observations of seedling die-back within the dense seedling assemblages (Fig. S2) are suggestive of an alternative hypothesis whereby the enriched OTUs represent pathogenic bacteria involved in seedling mortality. Alternatively, our observations could be explained as shifts in the saprotrophic proportion of the community in response to seedling death caused by species-specific pathogens below the detection limit of ARISA.

The involvement of soil pathogens in density-dependent seedling mortality has been reported previously via soil fumigation which was shown to reduce rates on seedling density-dependent mortality (Bagchi et al. 2014). Moreover, many generalized seedling diseases, such as damping off, can be caused by multiple soil bacterial as well as fungal pathogens (Lamichhane et al. 2017). The high beta-diversity between the three *C. angulata* seedling patches indicates that seedling density was promoting the abundance of different bacterial OTUs at each seedling patch. Possibly these data describe a non-species-specific response to *C. angulata* root exudate. It is also possible that different OTUs enriched at each patch represent non-specialist pathogens that function as host specialists (effective specialization) due to different host–pathogen–environment interactions, suggesting a role for opportunistic, non-host-specific

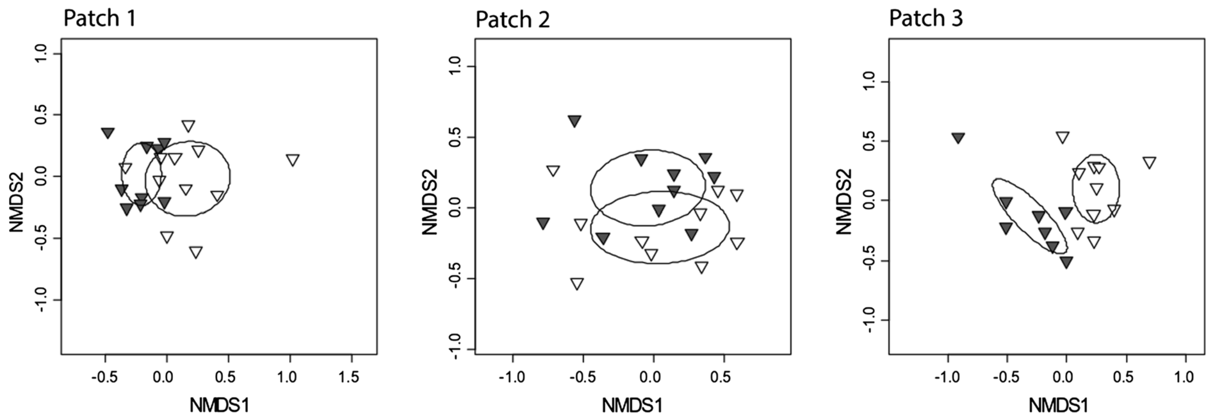

Fig. 3 NMDS ordinations of bacterial assemblages from replicate patches of geminating *C. angulata* (Patch 1–3). Colours are indicative of seedling density: grey = high-conspecific seedling density; white = low seedling density. Two-dimensional stress values range from 0.16 to 0.18. Stress values for fungal and oomycete ordinations were > 0.2 and are not depicted. Ellipses indicate group standard deviations

soil pathogens in maintaining rainforest diversity (Benítez et al. 2013).

There is evidence to suggest that an enrichment of bacteria with pathogenic traits is a feature that typifies rainforest soil communities. Studies comparing soil communities from undisturbed and cleared rainforests identified an enrichment of traits relating to virulence, disease and defence in the undisturbed rainforest soil communities (Mendes et al. 2015). This supports our hypothesized role for opportunistic, non-specific soil pathogens in seedling mortality.

Further investigations with a taxonomic approach, such as 16S rRNA sequencing, is required to reveal the identity and pathogenic potential of enriched OTUs. Additionally, as our field study of seedling patches was predicated on recruitment events coinciding with re-censusing efforts, we have only examined patch dynamics for one seedling species, *C. angulata*. Studies confirming our observations across multiple seedling species will be necessary in determining whether these plant–microbe interactions can impact mortality rates and plant diversity at the plot scale.

Although we did not detect evidence of species-specific pathogen reservoirs associated with adult trees, we observed repeated ecological patterns (bacterial communities clustering due to conspecific seedling density) across communities with high levels of beta-diversity. Our observation that microbial community changes, which may underpin seedling mortality, were present amongst seedling patches but not adult tree rhizospheres is consistent which previous research conducted at Davies Creek. Using the accumulated plant demographic dataset from Davies Creek, Green, Harms et al. (2014) demonstrated that the non-random mortality patterns, theorized to drive rainforest diversity, occur primarily in the smallest plant size classes (i.e. seedlings and small saplings). Moreover, Connell et al. (1984) demonstrated that proximity of seedlings to conspecific adults did not impact mortality rates, however, there was a significant impact of having a conspecific neighbour amongst seedlings and small saplings (Connell et al. 1984). Our observations and those form the literature lead to a tentative new hypothesis that opportunistic soil pathogens can facilitate non-random mortality patterns in tropical rainforests. Ultimately, manipulative or microcosm experiments are needed to link conspecific seedling density-driven changes in microbial community structure with seedling mortality and reveal microbial mechanisms that maintain rainforest diversity. Next-generation sequencing will be necessary to reveal agents of seedling mortality and identifying microbial indicators of rainforest soil function for use in conservation monitoring.

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Author contributions Experimental designs were conceived by JLW, PTG and AEF. Integration of long-term plant demographic data into microbial community sampling designs was carried out by KEH and PTG. Sample collection was carried out by JLW, PTG and AEF. Soil DNA extraction, Data QC, bioinformatics analysis, trait-based analysis and statistical tests of ARISA and rRNA were performed by JLW. Soil physicochemical analyses were carried out by CC JV and JLW. Manuscript was drafted by JLW and JLW, KEH, PTG, AEF, CC and JV contributed to the revision and copy-editing of the final manuscript.

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Microbial communities associated with distance- and density-dependent seedling mortality in a tropical rainforest – Supplementary data

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Running title: soil microbial communities in tropical rainforests

Key words: Janzen-Connell, rainforest diversity, soil pathogens, tropical

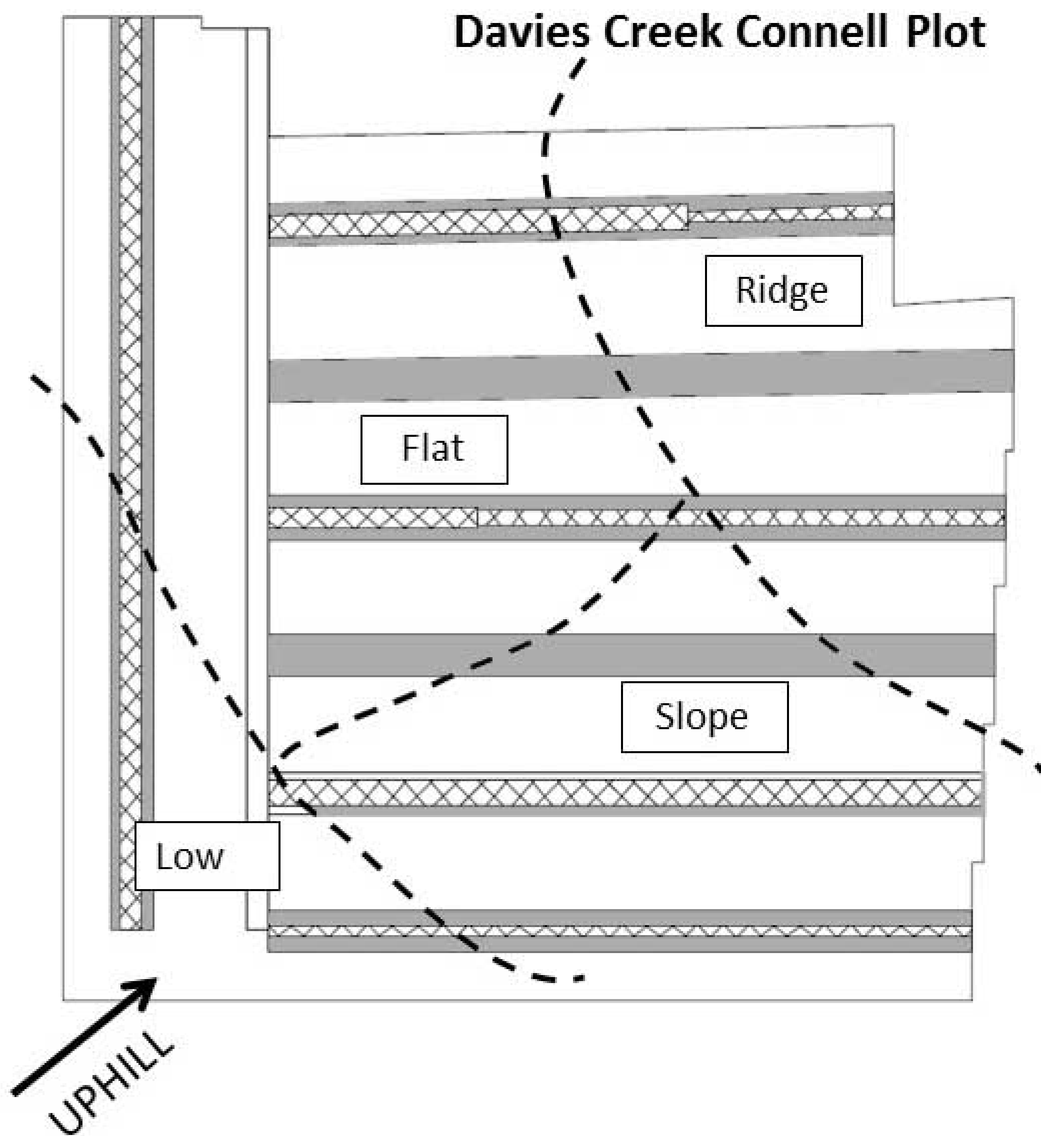

Figure S1 Map of Davies Creek. Dotted lines indicate the boundaries used to broadly classify areas of Davies Creeks as Flat, Ridge, Steep and Low for the purpose of comparing soil samples collected for this study. Demographic data have been collected for large trees across the whole plot since 1963; demographic data for medium trees have been collected within the shaded strips shown along the survey lines since 1963; saplings' and seedlings' demographic data have been collected from hatched areas along certain survey lines since 1963.

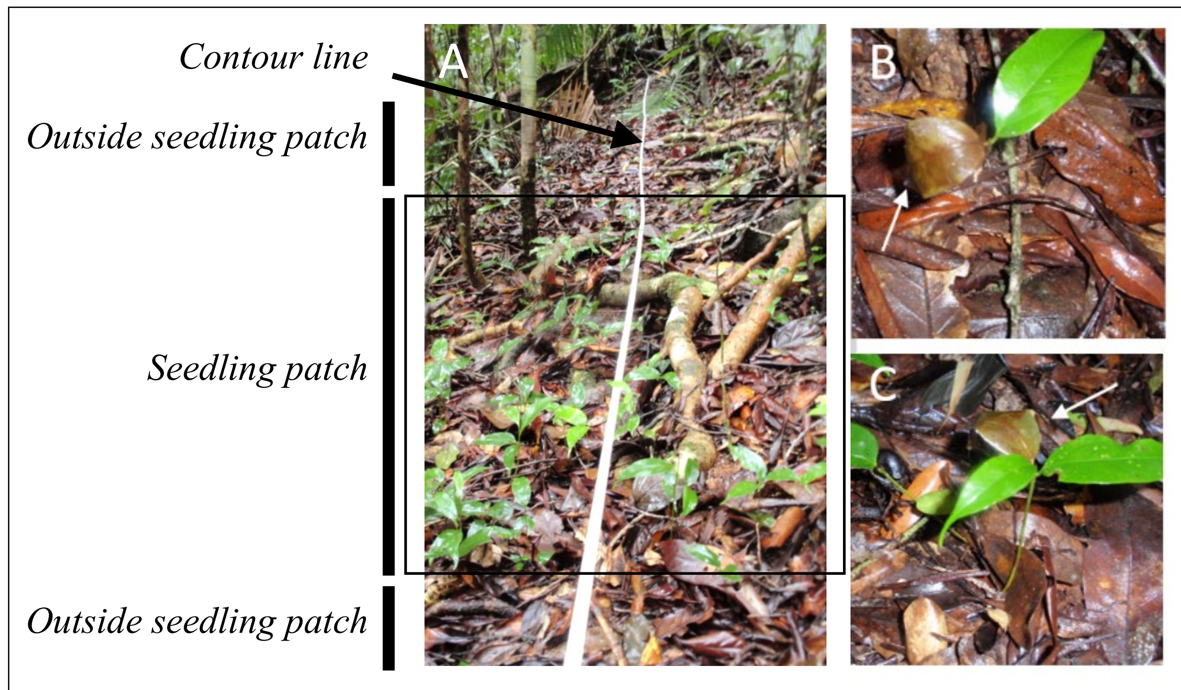

Figure S2 A: Figure illustrating the sampling strategy for conspecific seedling patches of *C. angulata*. Soil was sampled from within the seedling patch, and from two seedling-sparse areas, adjacent to the seedling patch, that fell along a line of constant elevation. B - C: Representative images of seedling dieback occurring within seedling patches

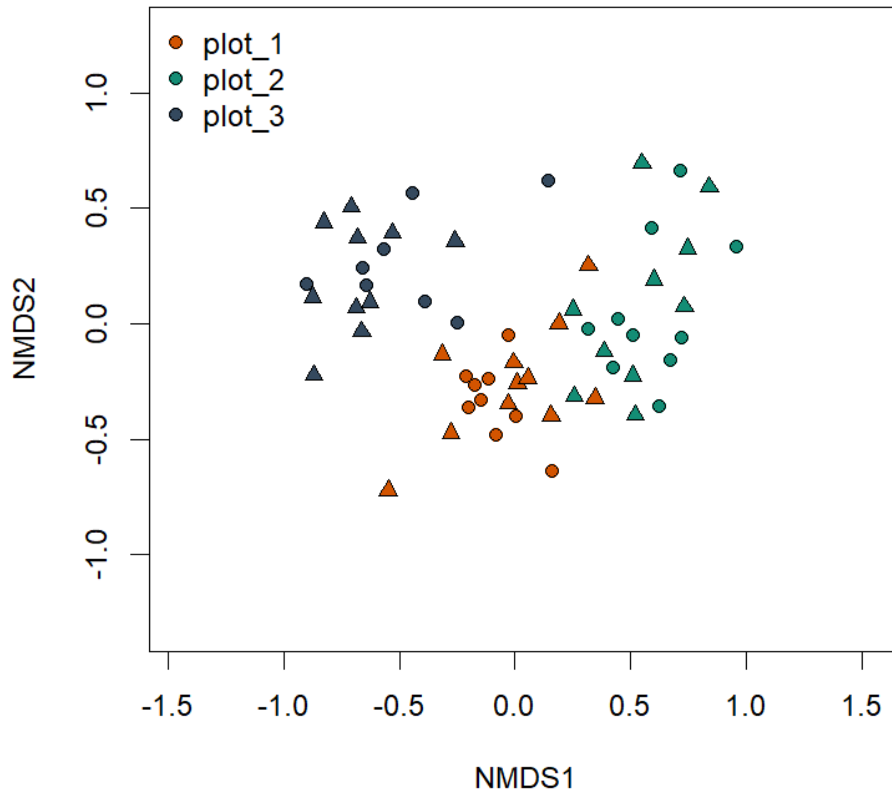

Figure S3 NMDS ordination of bacterial communities from replicate patches of geminating *C. angulata* (Patch 1-3). Colours are indicative of seedling patch, Shapes indicate seedling density: circle = high conspecific seedling density; triangle = low seedling density. Two-dimensional stress = 0.19 - 0.18.

Table S1: Distribution of seedling distances (m) from nearest conspecific adult tree for the 10 focal species used in this study

Species	N seedlings	Min distance	Max distance	Median distance
B. nitida	138	0.3	20.9	5.3
C. sublimis	828	0.2	33.4	6.9
C. succirubrum	6164	0.1	15.6	6.6
C. angulata	1575	0.2	30.1	6.5
E. wolfei	175	0.4	29.0	6.9
F. laurifolium	1260	0.4	13.8	7.3
Garcinia sp. nov.	350	0.4	32.2	5.9
Niemeyera sp. nov.	712	0.2	20.9	4.8
R. angustifolia	14056	0.0	12.8	4.5
S. endophloium	756	0.6	45.0	7.8