DIVERSITY AND HOST AFFINITY OF TROPICAL SEED-INFECTING BACTERIA

By

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Abstract

Numerous symbiotic relationships are known to exist between seeds and microbes, including both fungi and bacteria. Relationships between seeds and fungi play a crucial role in the survival of pioneer tree species during the seed-dispersal, seed-bank, and seed-germination phases. Prior studies have shown that these fungi can influence the establishment of pioneer trees, which in turn influences forest succession and recovery from disturbance. The question arises if similar roles are played by bacterial interactions in the seeds of pioneer species. The purpose of this thesis was to determine the diversity, host specificity of the bacterial community found in the seeds of three pioneer species in Barro Colorado Island, Panama. Six strains of bacteria were identified using molecular analysis methods focusing on the 16S rRNA gene. One was Gram negative, and the others were Gram positive. Four genotypes were unique relative to previous studies of bacteria in these tropical seeds. This expands our knowledge of the diversity of seed-associated bacteria. Two genotypes were found previously in an ongoing study of these seeds. Here, our findings expand our understanding of their host range, as here they were identified from tree species in which they had not been observed before. In future work we propose testing the effects of these bacteria on seed germination and viability. For now this work contributes to ongoing studies to understand the diversity, distributions, and host specificity of tropical microbes associated with plants at different life stages. Such an understanding will inform fundamental aspects of tropical forest ecology.

Introduction

Tropical rain forests are one of the most ecologically diverse ecosystems on Earth. They harbor a great number of macroscopic species such as plants and animals, for which they are most well known. However, rain forests also are home to an enormous richness of microorganisms (Torsvik et al. 1996, Dalling et al. 2010).

Many of these microorganisms have pathogenic relationships with plants (Torsvik et al. 1996). Accordingly, tropical plants and their seeds have developed numerous strategies to survive in pathogen attack (Dalling et al. 2010). Although plant pathology is relatively well understood for adult plants and saplings, there is little information about how seeds interact with the microorganisms in the soil before germination (Dalling et al. 2010).

Seeds of some tropical trees often can persist in soil for multiple years despite the presence of numerous pathogenic microbes (Gallery et al. 2007). Seeds of pioneer trees in particular form long-term soil seed banks in which small seeds can survive for long periods of time prior to germination. How they do so is unclear, but may reflect physical and chemical defenses against microbial attack, or the acquisition of beneficial microbial symbionts (Dalling et al. 2010). Understanding seed fate of pioneer species is important because pioneer trees are critical in forest regeneration and succession (Zalamea et al. 2015).

A large-scale experimental project was established at Barro Colorado Island, Panama to address seed fate in soil (Zalamea et al. 2015). In this experiment, seeds of 18 species of trees have been planted in five common gardens in the natural forest understory. The seeds were planted in mesh bags that allow microbial access but exclude herbivores, and were retrieved at intervals from 1 month up to 24 months. After the seeds were retrieved, they were assessed for viability, permeability, and germination, and evaluated for microbial infection (Zalamea et al. 2015).

Results to date have shown that seeds harbor both fungi and bacteria after soil contact (Zalamea et al. 2015). Previous work has addressed fungal communities of seeds after soil contact in this tropical forest (Gallery et al. 2007, Sarmiento et al. 2013), but relatively little is known about seed-infecting bacteria (but see Garcia et al. 2013).

The goal of this study was to examine the diversity, taxonomy, host specificity, seasonality, and distributions of bacteria in seeds of tropical pioneer trees. This research project focused on bacteria found in the seeds of three species: *Annona spraguei*, *Trema micrantha* (brown), and *Trema micrantha* (black). By identifying the bacteria inhabiting viable seeds of tropical pioneer species we hope to shed light on the possible symbiotic relationships that enable them both to survive and thrive in tropical forest soils.

Methods

We examined bacteria associated with the seeds of three pioneer species that persist in the soil seed bank of lowland forests in Panama. Prior to this thesis project, the research team collected seeds and buried them in the forest in Panama (Zalamea at el. 2015). The seeds then were examined for microbial infections, as described below. The 'seed collection and seed burial experiment' section describes work conducted by the team prior to this thesis project. The sections thereafter highlight the work done through this thesis project.

Seed collection and seed burial experiment

Mature fruits from at least five individual trees of each species were collected in 2012 and 2013 near Barro Colorado Island, Panama (BCI) by project personnel (see Zalamea et al. 2015). Seeds were removed from fruits immediately after collection and air-dried at 22°C under low red : far-red irradiance (Gallery et al. 2007).

Seeds from each maternal source per species were mixed thoroughly. Sets of 45 seeds per species then were mixed with 10 g of forest soil that had been sterilized by autoclaving at 121°C for two hours. Each set of seeds and soil was enclosed in a nylon mesh bag (pore size = 0.2 mm) that was then enclosed in aluminum mesh (mesh size = 2 mm).

Seed bags were buried in common-garden plots in the forest. Gardens were located in different forest- and soil- types (Zalamea et al. 2015). Garden sites had no adults of the study species within 20 m.

Seed bags were buried 2 cm beneath the soil surface and 40 cm apart (Gallery et al. 2007). Twelve bags per species were placed in each garden. After burial, leaf litter was placed back on gardens and was kept as intact and natural as possible throughout the experiment. More than ten species have been placed in these gardens. This thesis focuses on three of those species.

Seed bags were retrieved after various time intervals (see below). Ten seeds removed from bags, rinsed with tap water, and processed for microbial isolation. Surface-sterilization was done by successive immersion in 95% ethanol (10 sec), 0.7% sodium hypochlorite (2 min) and 70% ethanol (2 min) (Gallery et al. 2007; Kluger et al. 2008). Seeds were allowed to surface-dry after this treatment, and then cut in half under sterile conditions. One half of each seed was placed on 2% malt extract agar (MEA) in a 1.5mL micro-centrifuge tube for microbial isolation. These are referred to as ‘seed slants’ below, because the agar surface is slanted.

Culture tubes were incubated at room temperature (ca. 23°C) under natural light-dark cycles, and were scored for microbial growth for up to 2 years. Several bacterial strains were identified in these collections following the methods below (Garcia et al. 2013). This culture collection and the associated molecular data were expanded by this thesis project.

Bacterial isolation and identification

The samples studied here were obtained from the original seed slants of three species of tropical pioneer trees: *Annona spraguei*, *Trema micrantha* (brown), and *Trema micrantha* (black). *Annona* is in the Annonaceae, and *Trema* is in the Cannabaceae. *Trema micrantha* appears to represented two morphologically distinguishable species (here described as 'brown' and 'black' pending species descriptions).

Focal samples were chosen using two criteria: species of tree and time spent in the soil. For *Annona spraguei* and *Trema micrantha* (brown), the times chosen were no time in soil, one month in the soil and three months in the soil. For *Trema micrantha* (black) the times chosen were no time in soil, one month in the soil, three months in the soil, and six months in the soil. We then assessed which of these time points had sufficient bacterial cultures in the collection for analysis. Finally we focused on those bacteria that grew most effectively in culture. A total of eleven bacterial cultures were included in our final study. Cultures obtained from this process, their host plants, and the duration of burial in soil are listed in Table 1.

Table 1. Tree species, focal strains, duration of time in soil, and study plot of origin at Barro Colorado Island, Panama. PBXXXX are identification codes for each culture. For the burial durations, 1 month = 1 month in soil. 0 months = fresh seeds, which were not placed in soil. Plot names refer to common garden plots in the ongoing experiment. Although we initially chose a larger range of times, these strains grew most robustly and therefore are the focus of this thesis.

Tree species and strain IDs	Burial duration	Plot
Annona spraguei		
PB0623	0 months (Fresh)	N/A
PB0625	0 months (Fresh)	N/A
PB0631	0 months (Fresh)	N/A
PB0602	1 month	Pearson
Trema micrantha (brown)		
PB0688	1 month	Drayton
PB0690	1 month	Drayton
PB0708	1 month	Pearson
PB0735	1 month	Zetek
PB0744	1 month	Zetek
Trema micrantha (black)		
PB2095	0 months (Fresh)	N/A
PB2106	1 month	25-hectare

Microbial growth was extracted directly from the slant of the seeds and re-cultured on 2% malt extract agar under sterile conditions. The cultures were incubated at room temperature and observed daily for bacterial growth. Microscopy was used to confirm that the growth was bacterial, rather than fungal. From these cultures we chose those with the most robust bacterial growth, as indicated in Table 1.

Molecular analysis

For cultures identified to be bacteria, total genomic DNA was extracted utilizing the Y-PER Extraction method (Packeiser et al. 2012). A Qubit assay was done to quantify the amount of DNA present in the DNA extractions.

We then used the polymerase chain reaction (PCR) to amplify approximately 1000 base pairs of the 16S rRNA gene using the 1492 Reverse and 27 Forward primers. Two PCR recipes were used to compensate for different DNA concentations. The first PCR recipe that worked used 0.8 µL of each primer, 10 µL of Amplifier (AMP) solution, and 3.4 µL of PCR water (used for samples PB0625 and PB0602). The second PCR recipe was successful for the remaining samples, and consisted of 0.8 µL of each primer, 10 µL of Amplifier (AMP), and 7.9 µL of PCR water. The PCR conditions that resulted in positive results were: 1) 94^0C for 3 minutes, 2) 94^0C for the next 30 seconds,3) 55^0C for 30 seconds, 4) 72^0C for 1 minute then returned to step 2 and repeated the cycle 40 times, 5) 72^0C for 10 minutes, and 6) Hold at 4^0C.

To confirm the presence of DNA in the PCR product, gel electrophoresis was performed using SYBR green I stain in 1.5% agarose gels. Samples that showed strong, single bands of the appropriate length were sent for sequencing at the Arizona Genetics Core facility. Bidirectional Sanger sequencing was performed using the original PCR primers.

When sequence data were obtained, they were edited and assembled to form consensus sequences for each strain. The consensus sequences were compared against the NCBI GenBank database to identify each strain. We also compared these sequences against bacterial data obtained previously for the project to expand the culture and molecular database.

Results and Discussion

We first quantified the amount of genomic DNA from each culture. Results are shown in Table 2. We found a wide range of DNA concentrations from our different extractions, ranging over several orders of magnitude (Table 2). This variation likely reflects differences in bacterial taxonomy, with some species being easier to extract than others, perhaps due to secondary metabolites, pigments, or the nature of the bacterial cell walls. However, all strains yielded DNA using our methods (Table 2), indicating that our approach is useful for these tropical bacteria. The Y-PER method is inexpensive and appears to be effective at the DNA step.

Table 2. Assay DNA quantification results. dsDNA refers to double-stranded DNA.

Tree species and strain IDs	Qubit Assay (dsDNA)
Annona spraguei	
PB0623	$3.3x10^3$ ng/uL
PB0625	167 ng/uL
PB0631	40 ng/uL
PB0602	$6.32x10^3$ ng/uL
Trema micrantha (brown)	
PB0688	127 ng/uL
PB0690	104 ng/uL
PB0708	61.2 ng/uL
PB0735	181 ng/uL
PB0744	14.4 ng/uL
Trema micrantha (black)	
PB2095	127 ng/uL
PB2106	104 ng/uL

Overall, the diagnostic fragment of 1000 base pairs was successfully amplified using PCR for 10 strains (Table 3). Strains that were amplified successfully were sent for sequencing. Overall, six strains with successful PCRs were sequenced successfully (Table 3). The strains that were successfully sequenced

represented two bacteria from *Annona spraguei* and four from *Trema micrantha* (brown). We identified one bacterium from seeds representing time zero (no soil contact) and one month of soil contact in *Annona spraguei*. Bacteria identified from *Trema micrantha* (brown) were all obained from seeds that were exposed to soil for one month. Overall, two genera representing two phyla of bacteria were identified through this project: *Citrobacter* (Gammaproteobacteria), a Gram negative bacterium, and *Bacillus* (Firmicutes), a Gram positive bacterium (Table 3).

Successfully sequenced strains were compared with previously sequenced strains to determine if they were novel or had been recorded previously. The database for comparison had information of 460 bacterial isolates and 328 different bacterial strains (i.e., 16S genotypes) from the larger project (Garcia et al. 2013). The strains identified in this thesis project included four strains that comprise bacterial genotypes that were not previously recorded in the database (Table 3). The others were found previously in other host species, indicating that they appear to be host-generalists (Table 3).

Table 3. Results of PCR and sequencing for 16S. 'Yes' indicates successful sequencing. 'No' indicates failed PCR or sequencing. Notes are included for focal strains. N/A = not applicable. ND = not determined.

Tree species, strain IDs	Sequenced	Notes	Genus
Annona spraguei			
PB0623	No	N/A	ND
PB0625	Yes	Genotype found previously in *Apeiba membranacea* after 1 and 5 months in soil.	*Bacillus*
PB0631	No	N/A	ND
PB0602	Yes	New Genotype	*Citrobacter*
Trema micrantha (brown)			
PB0688	Yes	New Genotype	*Bacillus*
PB0690	Yes	Genotype found previously in *Trema micrantha* (black) after 6 months in soil.	
PB0708	Yes	New Genotype	*Bacillus*
PB0735	Yes	New Genotype	*Bacillus*
PB0744	No	N/A	ND
Trema micrantha (black)			
PB2095	No	N/A	ND
PB2106	No	N/A	ND

These results add to an already considerable diversity of the database in the Barro Colorado Island site in Panama: by adding only six new sequences we

increased our overall knowledge of bacterial diversity in seeds by adding four novel genotypes.

The bacteria identified through this project are of interest for future microbial ecology studies. *Bacillus* has been known to have strong positive effects on plant growth (Broadbent, 1977) and protection from pathogenic species (Kloepper, 2004). It is possible that these bacteria are recruited from the soil by seeds to promote early seedling growth. Similarly, *Citrobacter* can play a role in modulating the production of compounds relevant to plant growth hormones (indoles) (Lipsky et al. 1980). In future work, these bacterial strains could be applied directly to seeds in an experimental setting to determine their potentially positive roles.

Conclusions

Overall, this project contributes insight into the microbial diversity present in tropical soils and the portion of that diversity that associates with seeds in soil in a lowland forest. Analysis of six bacterial strains isolated in culture from seeds of tropical poineers yielded four novel genotypes and suggest a general pattern of host generalism for those strains found previously. Future work should address the potential costs/benefits of these distinctive bacteria and the degree to which they difffer in their effects, or are uniformly beneficial or costly, for different tree species. In this way a study of bacteria can complement previous work on fungi in studies of tropical forest dynamics. More generally such work can speak to the health of the ecosystem and might add to our understanding of pioneer species growth and forest regeneration strategies.

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