

Bacterial Richness of Soil Samples from Southern New Hampshire

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Summary

As DNA sequencing has become more popular, knowledge about the soil microbiome has expanded considerably. This technological advancement has led to a greatly increased understanding about the role of bacteria in soil. Previous studies have shown that, while geographic proximity plays a minor role in determining species richness (1), there exists a direct correlation between microbial diversity and both soil pH (1) and plant variety (2). In this paper, we examine the microbial content of soil samples taken from three locations in southern New Hampshire with varying pH and plant composition. We found that a minority of genera were shared between all three sites, and most genera were present in only one site, confirming the theory that soil microbes are site-dependent. The three shared, genera as well as the genera unique to specific samples, were paraphyletic. Our data suggests that pH is not the only factor affecting microbial composition, thereby confirming previous research and contributing to the understanding of the interaction between microbial communities and soil. While pH may affect microbial species richness (1), our results demonstrate that other factors, such as soil management techniques, play a larger role in the distribution of genera between different soil sites.

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Introduction

DNA sequencing is a taxonomic technique that allows scientists to identify species by genetic information. Its reliability and accuracy makes it a way to augment or possibly replace phenotypic analyses. Sequencing is becoming more powerful as the database of known sequences grows, and this benefits a variety

of applications ranging from discovery of new species, to customs security, to analysis of organisms' diets. However, it does have limits. For example, recent divergences can make distinctions between related species' DNA almost impossible (3).

Scientists have used the 16S rRNA gene as the primary region for bacterial analysis. The 16S gene plays a critical role in the manufacture of cellular proteins because it is a ribosomal subunit, and as such, it is highly conserved. A harmful point mutation would decrease the fitness of the bacterium, while a helpful mutation would be heavily selected for and come to dominate populations (4). Additionally, it is not thought to move via horizontal gene transfer. All these factors ensure its accuracy as a differentiator between species (5). Because the gene is only about 1,500 base pairs, it is relatively inexpensive and easy to sequence.

The 16S gene was used to rectify previous classification errors. In 1990, Carl Woese used 16S barcoding to show that the taxonomic groups that were then known as Prokarya and Eukarya could be more accurately divided into three domains: Bacteria, Archaea, and Eukarya (4). Woese's work played a central role in establishing the barcoding method and bringing it to prominence.

In this study, soil samples were collected from a blueberry farm, a home garden, and a temperate broadleaf and mixed forest in southern New Hampshire. Bacteria were cultivated from the samples, and 40 distinct colonies were subsequently chosen from each location for DNA isolation. The 16S gene was amplified and used for sequencing in order to identify the microorganisms through a BLAST search.

A study conducted by Robert Fierer and Robert B. Jackson asserts that soil pH has significant effects on microbial diversity while geographic proximity does not (1). The study compared the pH and bacterial diversity of soils from different climates and continents, including the Amazonian rainforest, savannah, tundra, and temperate forests. They discovered that microbial diversity is strongly correlated with soil pH, with more acidic sites

having the lowest diversity, and is not correlated with biome or location. Fierer and Jackson also noted that between two deciduous forest samples, both taken from the same forest in North Carolina, the soil with the higher pH had more bacterial richness than the other sample. In addition to pH, factors such as vegetation type, carbon availability, nutrient availability, and soil moisture may influence diversity at a local level (1).

Fierer and Jackson used culture-independent methods of measuring overall bacterial diversity. However, this study's method of species identification is culture dependent, using two types of minimal media: Rhizobium Defined Media (RDM) and R2A agar plates. These are both non-selective minimal media that support the growth of rhizobia and other fastidious bacteria that would be outcompeted on media with higher nutrient concentrations (5). The only difference between RDM and R2A is that the latter contains yeast extract, which favors slower-growing bacteria (5). Because of the nature of media (even non-selective media), only a small portion of the entire soil population will be cultivated (6). Despite this bottleneck, we hypothesize that the combined bacteria cultivated from both types of minimal media will still reflect the trends of bacterial biodiversity found by Fierer and Jackson.

On a broader scope, despite the samples being from the same geographic location, based on the soil pH and level of soil management, we hypothesized that a minority of species of the bacterial community would be shared between all three locations. Furthermore, we hypothesized that fewer species would be shared between all three locations than between any two locations and that fewer are shared between any two locations than are present in only a single location.

Scientists are realizing the potential impact bacteria have on everything from ecosystems to agriculture. According to an article by National Geographic (7), microbiologists are hunting for a certain bacteria that

could greatly enhance current agricultural methods. By sequencing the DNA of bacteria from our soil samples, we will know more about the bacterial community at the sites of our samples, we will be able to better understand what sequences differentiate and define species, and may even discover new species that could have medical, ecological, or agricultural applications.

Numerous studies have shown a correlation between increased bacterial diversity and increased plant growth and diversity; however, little is understood of exactly how microbial diversity affects plant flora. Gaining a better understanding of how diversity at the species level impacts ecological diversity is a key step to better and more efficient management, fertilization, and care of soils, especially for the production of food and the protection of lost and endangered environments. It is known that different bacterial species play unique roles in their microbial community. Certain bacteria control whether the soil is conducive or suppressive to diseases, while others have the unique ability to fix nitrogen in the presence of oxygen. Microbiologists believe these bacteria could revolutionize and improve current agricultural processes, making food production more efficient, consequently providing a tool to address world hunger (8).

This study hopes to discover, categorize, and analyze the bacteria found in our samples grown on two minimal media and to increase our understanding of the processes and interactions between microbes and their environment.

Results

To test our hypotheses, we investigated the microbial communities of soil samples from three different locations with varying pH levels. We collected soils samples from three locations (hereafter referred to as garden, forest, and blueberry farm), grew the microbial community on two types of selective media, isolated

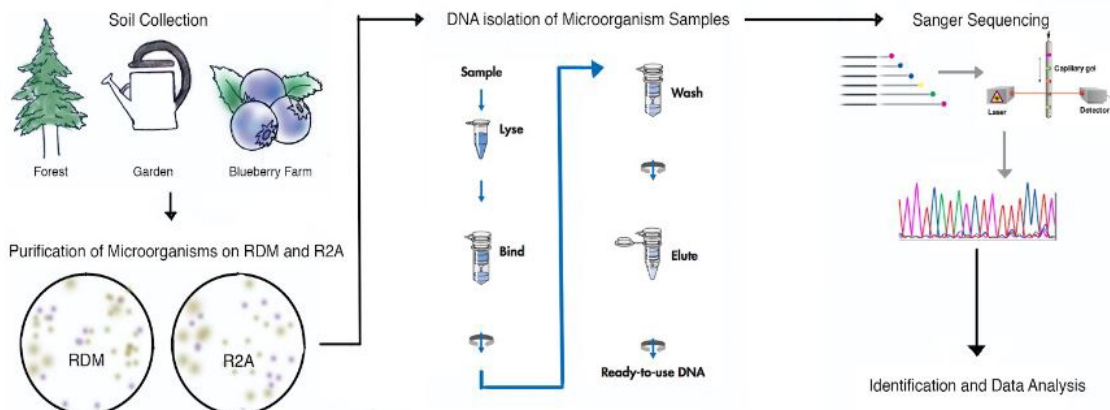


Figure 1. An overview of the experiment and data collection. The image demonstrating DNA isolation is from the Qiagen DNeasy kit protocol. The image demonstrating Sanger Sequencing is from biological illustrator Estevezj.

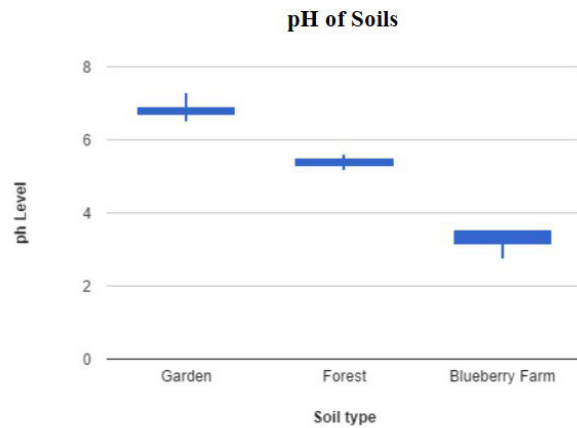


Figure 2. pH levels of all three soils. For each sample, we tested the pH multiple times (n=6 for garden soil; n=3 for forest soil; n=3 for blueberry farm soil). The replicates are measurement replicates, not biological replicates.

DNA from selected colonies, amplified the v3 region of the 16 rRNA gene, sequenced the amplicons, and used a BLAST search to identify the isolates. **Figure 1** summarizes the protocol we used.

Distribution of Shared Genera

Due to the various pH levels of the soils, we hypothesized that the samples would have a minority of genera in common (**Figure 2**). We organized our results in a Venn diagram (**Figure 3**), which allowed us to evaluate the distribution of shared genera.

At the genus level, we saw the highest level of richness in the garden, with 13 different genera identified. This supported the hypothesis that a more neutral pH (6.83) would yield a greater quantity of unique genera. However, this notion was not supported by the data collected from the blueberry farm and forest site. The blueberry soil had the lowest pH (3.28), yet exhibited a greater number of genera than the forest soil with the more neutral pH (5.39). There were only 7 different genera cultivated from the forest soil, while there were 10 unique genera cultivated from the blueberry patch. This suggests that other differences in soil types may play a significant role in determining the number of different genera in each environment.

The hypothesis that genera unique to one site would outnumber those shared between multiple sites was partially supported by the results described in **Figure 3**. Although a minority of genera were shared between all three sites, it is not true that fewer genera were shared between all three sites than were shared between any two or were unique to any one site. We observed a larger overlap in bacterial genera present for soils collected from the garden and blueberry farm than for any other sample pairing. The pH difference was the largest between the garden and blueberry soils, which suggests that they

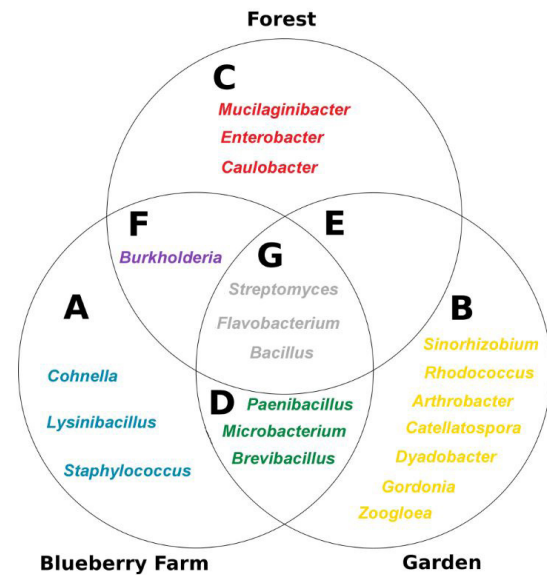


Figure 3. Venn diagram showing the genera present in each soil sample.

might share fewer isolates than either the garden and forest or blueberry farm and forest. However, our results show that there must be another factor that competes with the effects of pH in determining the number of shared genera between the three sites.

Evolutionary Relationships Among Isolates

The results prompted an inquiry into the relatedness of the genera within each soil sample. We found that soil type does not correlate with the evolutionary relatedness of bacteria (**Figure 4**).

In the cladogram, most of the genera, both unique and generalist, are paraphyletic. Of the two monophyletic clades observed, one was comprised of two genera shared by the blueberry farm and the garden soil (*Brevibacillus* and *Paenibacillus*), and one genera unique to the blueberry farm soil (*Cohnella*). The other contained a generalist bacteria (bacteria found in all soil samples) (*Bacillus*) and a genus unique to blueberry soil (*Lysinibacillus*). Therefore, it appears that genetically similar bacteria do not necessarily live in the same environment. The genus level cladogram shows that the bacterial genera present in all three soils (*Streptomyces*, *Flavobacterium*, and *Bacillus*) are not closely related phylogenetically. The fact that none of the overlapping genera found in two soil types form a monophyletic clade supports these conclusions. The genera unique to one soil also do not form monophyletic clades. For example, the organisms found only in the garden soil, the yellow genera, are spread throughout the cladogram. The forest soil, the red genera, and the blueberry farm soil, the blue genera, had fewer unique genera and also did not form monophyletic clades. Because the specialists

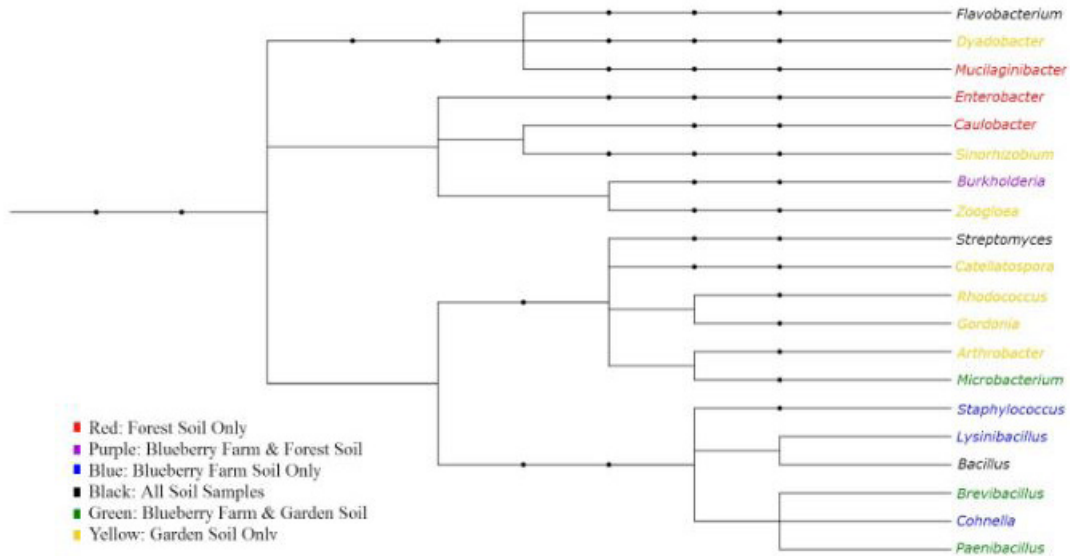


Figure 4. Cladogram of genera found in the experiment. The first two black nodes represent Archaea and Eukarya. The following black nodes represent different phylogenetic sub-groupings which show the phylogenetic difference between each genus. The cladogram was generated by the bioinformatics program PhyloT.

are genetically diverse, we can conclude that soil type and pH do not only select for genetically similar bacteria. Therefore, our data indicates that pH and soil type do not predict the genetic relationship between bacteria.

Frequency of Isolates in Different Soils

In addition to our other methods of data analysis, we looked at the contribution of different genera in the three soil samples. **Figure 5** shows the percentage of genera present in each sample location. *Streptomyces* and *Bacillus* make up over 60% of the isolates found in each soil sample. Our lack of species data makes it impossible to detect the species composition within the *Streptomyces* and *Bacillus* genera. All other genera

contributed a much smaller fraction of the total number of isolates identified.

Discussion

Our research resulted in three main findings. First, the garden soil contained the largest number of unique genera, thus making it the soil type with the most bacterial richness. Second, neither generalists nor specialists of any one soil type were closely related phylogenetically. Third, two genera (*Streptomyces* and *Bacillus*) were common in all three sites and made up a high percentage of the total bacteria identified. These conclusions were derived within the context of our experimental limitations.

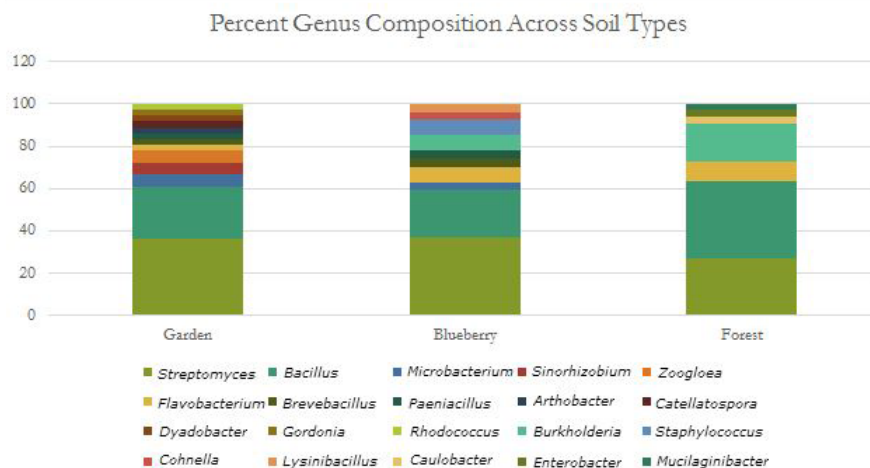


Figure 5. Stacked bar graph showing the relative frequencies of genera in the samples. Percentages were used as opposed to raw numbers to compensate for the fact that different soils yielded different numbers of samples.

It is noteworthy that section D (the overlap between garden and blueberry farm) of the Venn diagram contained the largest number of shared genera out of the three pairwise overlapping sections, even though sections A (blueberry farm) and B (garden) had the largest relative difference in pH. This might be explained by the fact that sites A and B underwent soil management. For example, the gardeners' use of inputs such as cow manure and seaweed might have introduced novel microbes into the environment that otherwise would not have been present at that site. We did not analyze our results with consideration toward the use of fertilizers, herbicides, irrigation methods, or crop rotation. Therefore, we would recommend another experiment analyzing the management factors that could have affected the bacterial composition of each soil environment. This may also provide a more thorough understanding of the impacts soil management has on bacterial communities.

We identified four possible explanations for the prevalence of *Streptomyces* and *Bacillus*. First, a single species of the genus *Streptomyces* or *Bacillus* could have high phenotypic variance and therefore have been classified as different isolates in our model. Second, the *Streptomyces* and *Bacillus* genera could include many generalistspecies that thrive in multiple soil environments. Third, these genera may include many specialist species that each thrives in their own environments. Fourth, the greater number of isolates from genera *Streptomyces* and *Bacillus* could be attributed to the nature of our media. It is possible that our R2A and RDM media were more conducive to culturing species of the *Streptomyces* and *Bacillus* genera, and thus we recovered a greater number of isolates from those genera.

There were several limitations to our experimental design. First, selection bias prevented an accurate and complete analysis of the bacterial communities. Rather than conducting a metagenomic survey of the community, we selected individual bacterial colonies based on unique phenotypes. Second, due to the limitations of using only nucleotide numbers 27–519 of the 16S rRNA for sequencing, not every isolate could be identified at the species level. Third, for a number of samples, the top results from NCBI were uncultured bacterial clones, which meant we could not identify the bacterium. Although our results at the species level supported our Venn diagram hypothesis, the data was rendered unusable for these reasons. These limitations also meant that it was impossible to conclude why *Streptomyces* and *Bacillus* comprised a majority of our isolates.

One final possible future study is to look into the role of nitrogen-fixing bacteria on the soils, and how the nitrate levels correlate with the microbial diversity of the

soil. N-free, semi-solid media could be used to isolate exclusively nitrogen-fixing bacteria, and nitrate levels in the soil could be measured (9).

As numerous studies have shown, the impact bacteria have on their environment and understanding how to maximize their potential for positive impact is paramount. Future work could contribute to the expansion of our knowledge and understanding of the mechanisms and relationships between bacterial fauna and overall soil health, which could in turn lead to more efficient fertilization and soil and crop management techniques.

Materials and Methods

Soil Collection and Site Identification

One half quart of soil was collected during the week of December 6, 2015 from three geographically distinct locations in Exeter, New Hampshire: an organic blueberry farm, a temperate broadleaf forest, and an organic home garden. Any litter was scraped aside from the surface and then soil was collected from the A horizon (the upper 2- to 3-centimeter range of topsoil).

Dilution of Soil

The experiment was initiated by diluting the soil samples. Four grams of soil were measured out and added to a test tube labeled 100, one of seven test tubes filled with 4.5 mL of water and labelled 10^0 through 10^{-6} . The sample was vortexed for one minute then left to rest. As shown in **Figure 6**, the soil was serially diluted by pipetting 500 μ L of the liquid above the settled soil from the 100 tube into the 10^{-1} tube, then 500 μ L of that solution into the 10^{-2} tube, etc., up until the final transfer into the 10^{-6} tube. Then five agar plates were labeled, either RDM or R2A. Concentrations 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} and 100 μ L of soil solution from tubes 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were pipetted onto the respective plates. Ten glass beads were placed onto the 10^{-6} plate and gently shaken horizontally, across the surface of the agar six or seven times. The plate was then rotated 60° and the shaking of beads was repeated, then another 60° and repeated.

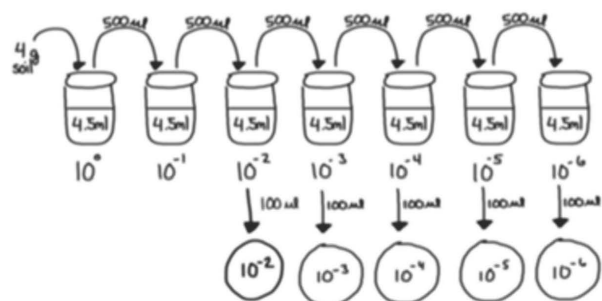


Figure 6. Illustration of serial dilution used to obtain distinct colony forming units on the agar plates.

The beads were then poured onto the 10⁻⁵ plate. The process was repeated for each consecutive plate. Lastly, the plates were incubated at 30°C for at least 72 hours.

Recipes for RDM and R2A Media

R2A and RDM media were made according to the recipes in *L. Microbiologist* (5). Both were autoclaved at 121°C for 20 minutes to sterilize before pouring into petri plates.

Purification of Microorganisms

The plates were split into two groups according to the type of agar used in the plate and incubated for 72+ hours. Following incubation, plates with between 30 and 100 colony forming units (CFUs) were chosen for analysis. Each plate was sorted and bacterial colonies were chosen if they had interesting or unique characteristics such as color, shape, surface texture, margin appearance, zone of inhibition, growth rate, metabolite evidence, or anything else worth noting. Colonies were not discriminated against based on their size. Colonies were also chosen so that they would be selected without contamination. Once colonies were chosen and numbered, they were transferred onto new plates. Each colony was streaked for isolation onto one quadrant of a plate. The plates were then inverted and incubated again at 30°C for 72 hours.

DNA Isolation

Qiagen's DNEasy Blood and Tissue kit was used to isolate gDNA for the purpose of amplification of the 16S gene. We used this kit because the protocol was designed for the purification of cultured Gram-negative bacteria.

Quantification of DNA

The extracted gDNA was then quantified with 50 µL of each sample being added to separate cuvettes. Using a UV/Visible light spectrometer (Thermo Electron Corporation Genesys 10uv), an absorbance value was produced based on the amount of nucleic acid material in solution. Since DNA absorbs light of a wavelength of 260 nm, this absorbance value was converted into a concentration (in ng/µL). Using the dilution formula ($C_1 \cdot V_1 = C_2 \cdot V_2$), the volume of gDNA solution needed to produce stock solutions was easily calculated.

**Note: In several cases, the volumes of DNA solution required to create the stock solutions exceeded the amount of solution. As a result, 10 ng of gDNA was transferred into the PCR tubes instead of a quantity in µL. This was calculated according to the formula:

$$10(\text{ng}) \div [\text{gDNA}](\text{ng/ml}) = V_{\text{DNAsoln}}(\text{mL})$$

The measured value was then converted into concentration (mg/mL) using Beer's Law, which was then converted to ng/µL for ease of use.

PCR

The next step after quantifying the DNA was to prepare each sample for PCR using 0.2-mL PCR tubes. We added 25 µL of HotStarTaq Master Mix containing Taq and dNTPs, 1.2 µL of 27F primer, and 1.2 µL of 1492R primer to each PCR tube. The DNA concentration data was used to determine how many microliters of each DNA solution contained 10 ng of DNA. Once this quantity of solution was added, molecular grade water was added in order to make the total volume 50 µL in each PCR tube.

For certain products that had more than 10 ng/µL, stock solutions were made to adjust their concentration to 10 ng/µL. Then, 1 µL of stock solution was added to 21.6 µL of molecular grade water along with the Master Mix and primers (**Table 1**).

The conditions for the thermocycler (PCR reaction) were:

1. 95°C for 15 minutes – activate the *Taq* polymerase
2. 94°C for 45 seconds – denature DNA
3. 50°C for 45 seconds – anneal primers to DNA
4. 72°C for 1.5 minutes – elongate (replicate) DNA
5. Repeat steps 2–4 29 times.
6. 72°C for 10 min. – final elongation

Gel Electrophoresis

PCR was confirmed with gel electrophoresis. In order to determine the success of each PCR reaction, 0.8% agarose gels were made by adding 0.8 grams of agarose to 100 mL of SYBR safe solution in 0.5X TBE. Heating and stirring intervals were repeated until the solid agarose powder had completely dissolved. The solution was allowed to cool to 60°C, and then poured into gel trays. We combined 10 µL of each sample with 2 µL of loading dye in a 0.2 mL tube and mixed. Lastly, 10 µL of the PCR-loading-dye solution was loaded into corresponding wells in the gels and allowed to run. 1X TBE was used as a running buffer, and gels were run at 12 volts/cm. If a defined band appeared, we knew PCR had been properly carried out. We expected band lengths around 1465 bp.

1492R	GGT TAC CTT GTT ACG ACT T
27F	AGA GTT TGA TCM TGG CTC AG

Table 1. Primers used to amplify a portion of the 16s rRNA gene.

After the gels were run, PCR was redone for products that produced fainter bands. Those with stronger bands were set aside for cleaning in the next step. Then, we ran the gels again to make sure there was actually DNA present.

Cleaning PCR Products

After confirming the presence of DNA with gels, we cleaned our PCR products using QIAquick PCR Purification kits. The QIAquick PCR Purification kit filtered out primers, nucleotides, polymerases, and salts from the PCR.

Sequencing

After obtaining clean DNA samples, each sample was prepared for the sequencing reaction. This required a second quantification step. A blank cuvette containing 50 μ L of Buffer EB was used to blank the UV/Vis Spectrophotometer. We added 10 μ L of purified PCR product and 40 μ L of Buffer EB to a cuvette and placed it in the spectrophotometer. The spectrophotometer was blanked every two absorbance readings. The absorbance of 260 nm light corresponded to the concentration of purified PCR product in the cuvette. This value was used to calculate how many μ L from each sample were needed to create stock solutions with 7.5 ng DNA/ μ L stock solutions for samples of high enough concentrations.

Samples were prepared for the sequencing reaction using the 7.5 ng/ μ L stock solutions. We combined 5 μ L of stock solution with 5 μ L of molecular grade water in a 0.2 mL PCR tube. For samples with concentrations too low to make stock solutions, calculations were made to determine the volume necessary to transmit 30 ng of DNA into the PCR tube. Enough molecular grade water was added to each of these samples to raise the total volume to 10 μ L. Samples were then submitted to GeneWiz. GeneWiz carried out the sequencing reaction and subsequently sequenced the DNA samples.

The conditions for the thermocycler (sequencing reaction) were:

1. 96°C for 1 minute – initial denaturation
2. 96°C for 10 seconds – DNA denaturation
3. 50°C for 5 seconds – primer annealing
4. 60°C for 4 minutes – extension
5. Repeat steps 2-4 25 times.
6. 4°C hold

Determining Identity of Isolates

We used an NCBI BLAST search querying the nucleotide collection (nr/nt) database using default settings to identify the isolates. We identified genera based on the combination of lowest E-value, highest Max Score, and highest Total Score.

Measuring Soil pH

Vernier probes were used in this experiment to measure the pH of each of the soil samples. Each probe was calibrated with the pH of two known solutions with a pH of 7 and 10. Each sensor was first rinsed thoroughly with deionized water, then carefully suspended (without touching the bottom of the beaker) in the pH 7 solution and allowed to stabilize before equating the voltage reading with pH 7. After a second rinsing with deionized water, the probe was placed into the pH 10 solution and again allowed to stabilize before equating the measured voltage with pH 10. The probe was then ready for use on the soil sample solutions.

To test the pH of the soil, one sample was taken from each location (blueberry farm, forest, and garden) and then 50 g samples were taken from each soil sample and placed in separate beakers (n=6 for garden soil; n=3 for forest soil; n=3 for blueberry farm soil). We added 100 mL of distilled water to each beaker, and the mixture was stirred. Each mixture was then stirred every three minutes for fifteen minutes. After the final stirring step, the mixtures settled for five minutes before pH was tested with the vernier probes.

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