Effect of Diet on Bean Beetle Microbial Communities

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Interest in microbiomes has increased greatly over the last several years. Studies of microbiomes involve the integration of laboratory and analysis techniques from a range of sub-disciplines and allow the infusion of ecology into introductory biology laboratories while using molecular techniques. In this exercise, students plate homogenates of bean beetles reared on different host bean types onto different media. They then describe the colonies that are formed to estimate taxonomic diversity. The students also extract DNA from a subset of colonies, perform PCR for the 16s rRNA gene, and analyze the sequence data using BLAST to further identify bacterial species. Finally, students are given data from MiSeq analysis in order to examine the effect of diet on both culturable and unculturable microbiome communities. This final step allows students to work with large datasets.

Keywords: bean beetles, microbiome, PCR, bioinformatics, species diversity, community ecology

Introduction

Complex interactive biological systems and large datasets are increasingly central to undergraduate training. Studies of microbiomes can be a wonderful way to introduce students to all of these concepts and have the added benefit of bringing in exciting and current real-world research to the undergraduate lab course. The microbiome (collection of genomes of microbes within a system) and microbiota (collection of organisms within a system) are increasingly in the news as scientists begin to tease apart how the system of microbes within an organism interacts with and affects the larger organism. Scientists are beginning to connect gut microbiota to diet and overall health. This field of study is relatively new and accessible within an undergraduate laboratory.

In insects, gut microbiota vary widely among species (Engel and Moran, 2013). These microbial communities have been found to affect immune response, protection from pathogens and parasites, nutrition, and communication (Engel and Moran, 2013). In addition to gut microbiota influencing multiple aspects of the biology of insects, the biology of insects can have a reciprocal effect on the structuring of the microbial community within the insect. For example, across species, diet has been shown to influence the microbial community in insect guts (Coleman et al. 2012).

Bean beetles (*Callosobruchus maculatus*) are a widely used model system for inquiry-based laboratory education because they are easy to rear (Beck and Blumer, 2007). They are a stored-product pest that infests a variety of dried bean species that differ in nutrient make up and in plant secondary compounds (Laudick and Beck, 2016). As a result, diet has the potential to affect the microbial community within the beetles.

We have used this experiment during the second semester of our introductory biology sequence. The laboratory course is a stand-alone two credit course in which students carry out several multi-week research modules related to the research interests of faculty at Emory University. This research module was introduced as a replacement for a single-week PCR lab. In addition, the research module allowed the introduction of ecological concepts related to community structure in our introductory sequence that otherwise does not cover ecology.

Student Outline

Reading Before the 1st Lab Period

Introduction to Bean Beetles:

Bean beetles (cowpea seed beetles), *Callosobruchus maculatus*, are agricultural pest insects of Africa and Asia. Females lay their eggs on the surface of beans (Family Fabaceae). Eggs are deposited (=oviposition) singly and several days after oviposition, a beetle larva (maggot) burrows into the bean. At 30°C, pupation and emergence of an adult beetle occurs 21-30 days after an egg was deposited. Adults are mature 24 - 36 hours after emergence, and they do not need to feed. Adults may live for 1-2 weeks during which time mating and oviposition occur. Since larvae cannot move from the bean on which an egg was deposited, the oviposition choice of a female determines the future food resources available to her offspring (Brown and Downhower, 1988). It is the most critical choice a female makes for her offspring because it will influence their growth, survival, and future reproduction (Mitchell, 1975; Wasserman and Futuyma, 1981). Although females can be induced to lay eggs (oviposit) on a wide range of bean species, very few bean species result in normal development and the successful emergence of adults. Some bean species are very clearly toxic to *Callosobruchus maculatus* larvae (Janzen, 1977).

Research Question:

How does diet and nutrition relate to our internal microbe populations (our microbiome)? Does diet impact the species present inside our bodies? Does diet impact the proportion of various species within our bodies?

Experimental Approach:

You will explore the microbiota of bean beetles raised on different beans through several complimentary approaches in order to assess how diet may affect the bean beetle microbiota. First, you will culture the microbiota of bean beetles and phenotypically describe the resultant microbe colonies. Second, you will select a microbe from these cultures to identify via DNA amplification and sequence analysis. Third, you will have access to a large dataset of sequencing reads of the complete microbiome from bean beetles reared on two different host beans.

Student Handouts for Each Lab Period

Culture of Bean Beetle Microbiota (Lab 1):

Prior to lab, beetles were sacrificed by freezing them at -80°C for 5 minutes. Before collecting and plating the internal microbiota of the beetles, you should sterilize the outside of the beetles to avoid growth of external or contaminating microbes.

- Using sterile tweezers to grasp the beetle, submerge it in the following solutions:

  o Bleach for 3 seconds

  o Sterile water for 10 seconds

  o 100% ethanol for 5 seconds

  o Sterile water for 10 seconds

- Place the beetle in a sterile microtube and add 450 μL sterile saline solution.

- Using the sterile pestle, crush the beetle to release its interior microbiota.

- Pellet the large debris by spinning in a microfuge for 5 seconds.

- Plate 100 μL of the microbiota solution on each of the following plate types:

  o Nutrient agar (NA)

  o Eosin Methylene Blue (EMB)

- o   Blood Agar (BA)

- o   Phenylethyl alcohol agar (PEA)

- The solution will be absorbed by the PEA plate quickly, so spread this plate immediately after pipetting.

- Dilute 20 µL of the remaining microbiota solution by mixing with 180 µL of sterile saline and plate 100 µL of this onto an NA plate.

- Repeat this process for beetles raised on the other bean types.

- Plate 100 µL of your sterile saline solution on each of the four plate types to serve as negative controls.

- Store plates at room temperature for one week.

Phenotypic Analysis of Bacterial Diversity and Richness (Lab 2):
    We want to estimate the species diversity and richness for the microbiota of bean beetles grown on different bean types. Use careful observation to describe the visual appearance of the cultured microbes and to count them.

1.   Describe the unique microbial phenotypes you can observe and record the number and proportion of colonies with each phenotype for each plate. Use the notations below for your descriptions.

     Color: W (white), O (off-white), R (red), O (orange), Y (yellow), B (brown)

     Gloss: S (shiny), M (matte)

     Form: C (circular), I (irregular), F (filamentous), R (rhizoid)

     Elevation: R (raised), C (convex), F (flat), U (umbonate), Cr (crateriform)

     Margin: E (entire), U (undulate), F (filiform), C (curled), L (lobate)

     For description or images of morphology types see http://microbeonline.com/colony-morphology-bacteria-describe-bacterial-colonies/

2.   Use your observations to estimate the total number of species cultured from each beetle.

3.   For each predicted unique species, estimate its relative richness in the community.

Amplification of DNA
    Each student may select 1 microbial colony to study via DNA amplification and sequence analyses in order to identify its species. You may coordinate with your lab partners and other groups in order to best sample your microbial phenotypes.

The order you will follow is:

1.   Assemble the PCR master mix on ice.
2.   Aliquot 15 µL of the PCR master mix to 6 tubes of a labeled PCR strip (leave the other 2 tubes empty).
3.   Pick a bacterial colony.
4.   Mix the bacterial colony in water (henceforth, colony suspension).
5.   Add 10 µL of the colony suspension, positive control DNA, or water to the correct PCR tube that contains the PCR master mix.

Specific instructions for each step:

1. **Assemble the PCR master mix on ice.**
   a) Be sure all reagents thaw completely before starting. **Keep reagents on ice once thawed**. Vortex and then spin each reagent briefly (for small tube spin inside of an empty 1.5 mL tube). Place each reagent back on ice.

   b) In a 1.5 mL microcentrifuge tube, add reagents in the order listed in the table below. Check-off as you go to be sure all reagents are added.

   c) After all reagents are added, cap the tube, vortex briefly to mix, spin briefly to force the liquid to the bottom of the tube, and return the tube to ice.

Reagent	1X	7X	Check					
dH$_2$O	5.75	40.25						
10X PCR Buffer	2.5	17.5						
50X dNTPs	0.5	3.5						
10X Forward Primer (27F)	2.5	17.5						
10X Reverse Primer (1429R)	2.5	17.5						
Taq polymerase	1.25	8.75						
Colony suspension	10.0	**Add separately to each PCR tube**						
Total (µL)	25	105	N/A					

2. **Aliquot 15 µL of the PCR master mix to 6 tubes of a labeled PCR strip on ice.**
   a) Label the PCR strips. When you get the DNA sequencing results, you need to have all the information about the colony you sequenced. In your notebook, write down the order of reactions on your PCR strip including plate type and colony morphology. Also note the location of your positive and negative controls.

   b) Add 15 µL of the PCR master mix to the bottom of each tube.

   c) Cap the tubes and keep them on ice.

3. **Pick a bacterial colony.**
   a) Get 4 sterile 1.5 mL microcentrifuge tubes and label each.

   b) Add 100 µL of sterile dH$_2$O to each tube.

   c) Set a P20 Pipeteman to 10 µL.

   d) Attach a sterile yellow tip to the end of the P20 Pipeteman.

   e) Gently touch the pipette tip to a well-separated colony from one of the bacterial growth plates. (Bacteria will stick to the pipette tip.)

4. **Mix the bacterial colony in water (suspend).**
    a) Submerge the pipette tip in the appropriate tube of 100 μL water and pipette up and down vigorously for about 5 seconds to suspend the colony.

5. **Add 10 μL of the colony suspension to a tube that contains the PCR master mix.**
    a) Using the same pipette tip that was used for picking and mixing, transfer 10 μL of the colony suspension to the master mix in the appropriate PCR tube.

    b) Pipette up and down 3 times to be sure the colony suspension and PCR master mix are well-mixed.

    c) Keep the PCR strip on ice.

    d) Go back to step 3d and repeat until one colony from each plate has been picked, suspended, and the suspension added to the correct PCR tube.

    e) For the positive control, add 10 μL of the positive control DNA to the PCR tube labeled for positive control. For the negative control, add 10 μL of sterile water to the PCR tube labeled for negative control.

    f) Cap the PCR strip so all tubes are sealed. **Keep your assembled reactions on ice**.

    g) Tell the laboratory instructor you are finished assembling your PCR reactions.

Your instructor will collect your tubes to be placed in the thermocycler. The thermocycler will run the following program and will have your samples for your next lab period:

95ºC, 10 min (purpose is to help disrupt bacterial cell walls/membranes to release DNA)

Then 36 cycles of:

95ºC, 30 sec

55ºC, 30 sec

72ºC, 1.5 min

Lastly,

72ºC, 4 min

4 ºC, hold

Visualizing and SequencingoOf DNA (Lab 3)
1. Pour an agarose gel

    a. Assemble your gel box and comb – make sure gaskets are in place.

    b. Bring your agarose to the instructor to have 7.5 μL Sybr Green added.

    c. Swirl to mix well.

    d. Pour in your gel box.

e. Pop any bubbles.

2. Prepare your samples

a. First mix 2 µL PCR reaction with 198 µL water (save this for later sequencing).

b. Mix 7 µL 5X DNA loading dye to remaining PCR reaction .

3. Load gel

a. Load 7 µL premixed DNA ladder into the leftmost lane.

b. Load 20 µL of your prepared samples into lanes skipping one lane between each sample.

4. Run your gel at ~135 V until the dye front is more than halfway through the gel.

5. Visualize your PCR reactions using the UV box.

6. Successful PCR reactions should load 10 µL of your reserved-for-sequencing sample into your assigned well in the 96-well plate.

7. Your instructor will add sequencing primers and send the plate out for sequencing.

Analyzing Sequencing Results (Lab 4)
*remember that these analyses should be recorded in your lab notebook too.

1. Download your .pdf and .seq files for your PCR reaction. If your PCR reaction (or sequencing reaction) failed, then download a different sequencing reaction. Record the length of your sequence in your .seq file here:

2. Open your chromatogram and determine where to trim your sequence. Record the starting and ending base number of your trimmed sequence here:

3. Now go to blast.ncbi.nlm.nih.gov/Blast.cgi. Select nucleotide blast, and paste in your sequence. To the right of your query sequence, enter in your starting and ending base numbers into the query subrange fields. In the 'choose search set' area click 'other' and then select '16S ribosomal RNA sequences.' Click the blue BLAST button to run your search (it may take a few minutes to complete). Analyze your results:
    a. Do you think you can identify your bacterial species? If so, what species do you think your bacteria was?

    b. Is there any ambiguity in your identification (i.e., might your bacteria be one of several hits returned by the BLAST search)?

    c. For your best matching sequence, is it a perfect match? What is your percent identity and coverage?

    d. Discuss your results and reasoning with your lab partners and then with your lab instructor.

4. Research your identified species:
    a. Is it gram-negative or gram positive? Does this make sense given the plate you isolated it from?

    b. Where is your bacterium normally found?

    c.   Can it cause infection?

    d.   Is it known to exist in gut microbial communities?

    e.   Any other useful information?

Analyzing MiSeq Microbiome Diversity Data (Lab 5)

        We isolated DNA from individual male and female bean beetles that had been reared on either mung beans or black-eyed peas and then surface sterilized using the same procedure that you used in lab. The DNA samples were then sequenced for the 16S rRNA gene using a MiSeq protocol (www.illumina.com/systems/sequencing-platforms/miseq.html). The data from the sequencing runs was processed through a bioinformatics pipeline called "Mothur" (www.mothur.org, Schloss et. al. 2009) to identify the different bacterial species in each sample. The bacterial species are identified as OTUs (operational taxonomic units). In our samples, 1543 different OTUs were identified. In the spreadsheet, one sheet gives the relative abundance of each OTU in each sample, another sheet gives you the identification of the OTU, and the last gives the square of the frequency for each of the top 50 OTUs in each beetle (helpful for Inverse Simpson's index calculations in Q5). Use these data to answer the following questions.

1.   What are the most abundant bacterial species in the male/female and black eyed/mung beetles?

2.   Do the most abundant species differ based on host bean type or between males and females?

3.   Is your sequenced bacterium (that you cultured) present in the MiSeq dataset? If so how abundant is it?

4.   Given your comparisons of the different approaches to identifying microbiome communities, which approach seems to be more reliable and why?

5.   Using the top 50 most abundant OTUs, determine whether the Inverse Simpson index differs based on host bean type (Inverse Simpson's index = $1/sum(\text{species frequency}^2)$ – use the sheet that has already calculated the square of the frequencies for the top 50 OTUs for you. Because you are comparing two host types, you should use a 2 sample t-test (in-silico.net/tools/statistics/ttest). Does bean type have an effect on the Inverse Simpson's index? What was your p-value?

6. Now compare the Inverse Simpson's indexes between males and females. Consider the data from each host bean species separately.
    a. P-value and interpretation for mung:

    b. P-value and interpretation for black-eyed pea:

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Materials

Materials are for 1 lab section of 24 students working in 6 groups of 4

Lab 1
Six freeze-killed beetles reared on different host types (available from Ward's)
Six sterile tweezers
Six 15-mL conicals of 10% bleach solution
Six 15-mL conicals of 70% ethanol solution
12 15-mL conicals of 0.9% sterile saline
Sterile Eppendorf tube with pestle
Six 1.5 mL tubes sterile saline solution
30 Nutrient agar plates (Difco, #211665)
12 EMB plates (Fluka, #62087)
12 PEA plates (Criterion, #C6611)
12 Blood agar plates (Teknova, #T0134)
spreaders, alcohol dishes and Bunsen burners

Lab 2
Six ice buckets
36 PCR tubes
Six 1.5mL tubes sterile molecular grade water
Six tubes 20 µL 10X Taq buffer
Six tubes 20 µL 2 uM forward primer – 27F (5' - AGA GTT TGA TCC TGG CTC AG)
Six tubes 20 µL 2 uM reverse primer – 1492R (5' - GGT TAC CTT GTT ACG ACT T)
Six tubes 20 µL 2mM dNTPs
Six tubes 20 µL diluted TAQ
Six tubes 5 µL template DNA
One PCR machine

Lab 3
Six gel apparatus
Three gel power supplies
Melted 0.8% agarose in warm water bath
Instructor held Sybr Green
6 L running buffer
Six 1.5 mL tubes 5X DNA loading dye
Six tubes DNA ladder 1 kb
One 96 well sequencing plate
Sequencing primer for instructor
UV box

Labs 4 & 5
Student access to computers
Shared online Google Forms for data submission (see Appendices C and D).

Notes for the Instructor

Details on the culture and handling of bean beetles are available at the bean beetle website (www.beanbeetles.org). Information on accessing and submitting student data can be found at www.beanbeetles.org/microbiome and https://www.beanbeetles.org/microbiome/microbiome-data-and-metadata-guidelines/.

In lab 1, instructors will need to introduce the module to students with a discussion about microbiota and how they relate to diet and health. The difficulty of culturing unknown microbes should also be a point of discussion, and a comparison of cultured microbiota grown in aerobic vs anaerobic conditions could be a nice addition to this lab exercise. For this lab session, plates will need to be prepared ahead of time. We pour our own nutrient agar, EMB, and PEA plates using the directions on the container. The blood agar plates are ordered pre-poured.

Before or during lab 2, students should be introduced to the concepts of species diversity and species richness. Students will likely struggle with the phenotypic classification of their microbe colonies, so instructors may need to ease student anxiety with the sometimes ambiguous nature of observation. Example calculations of diversity and richness should be demonstrated on the board. Instructors should also help students gauge which of their NA plates to use as different groups may find very different concentrations of microbes. Plates should be disposed of in biohazard waste. For the PCR reactions, instructors should have students leave their samples on ice with no polymerase added until all groups are ready to add the polymerase. Instructors should record the layout of samples in the PCR machine as labels can come off during the reactions.

In lab 3, it can be helpful to provide gels for students to practice loading dye into before having to load real samples into their own gels. We use SybrGreen instead of ethidium bromide as it is a safer alternative but we still take precautions such as having instructors add the dye to the gels before pouring, using gloves when loading or handling the gel, and disposing of gels in chemical waste containers. Colony PCR is a somewhat tricky procedure, so instructors should be prepared for some failure at this step. For successful PCR reactions, we use Eurofins for sequencing (https://www.eurofinsgenomics.com/en/home.aspx).
Sequencing results are typically ready within a day from when the samples arrive at the sequencing facility. They will accept samples in tubes or in a 96-well plate. The cost is approximately $5 per sample for clean-up and sequencing.

In the computational labs (labs 4 and 5), it is useful if students are able to bring their own laptops. Students viewed chromatogram files in standard PDF

format prior to BLAST searches. An alternative is to have students view the chromatograms and quality data in a viewer such as SnapGene Viewer (http://www.snapgene.com/products/snapgene_viewer/). Sequences can be trimmed in SnapGene Viewer and then exported as FASTA files. Bacterial species also can be identified using a curated database of 16s rRNA sequences such as Silva (https://www.arb-silva.de/aligner/). Instructors should help coordinate data dissemination between groups of their sequencing results (for example a Google form where students can enter the identified species, their sequence, and the phenotypic characterization of the original colony). A prelab assignment is provided in Appendix B.

In our introductory biology laboratory course, the students do not carry out DNA extractions for MiSeq analysis of 16S rRNA. In Appendix A, we include the DNA extraction protocol if you want to implement this extension in an upper-level course or conduct the analysis yourself so that you can provide the data to your students for analysis. Most sequencing facilities will not sequence a small number of samples. However, Mr. DNA (http://www.mrdnalab.com/16s-ribosomal-sequencing.html) will do so for $80 per assay. They will even carry out the microbiome bioinformatics analysis on request. The metagenomic data that we provide to our students is available in the Supplemental Materials.

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Acknowledgments

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Dr. Megan Cole is a Lecturer and Director of Undergraduate Laboratories for the Department of Biology at Emory University. She has B.A. degrees in Biology and Computer Science from Amherst College and received her PhD in Biology from MIT where she studied genomics of embryonic stem cells. She did her postdoctoral training at Georgia Tech and Emory University where she was a Fellow in Research and Science Teaching as well an NIH NRSA Fellow. In her current position, she develops authentic research experiences for undergraduates in introductory biology lab courses by bringing Emory Faculty research into the teaching labs.

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Dr. Christopher Beck is a Professor of Pedagogy in the Department of Biology at Emory University, where he teaches an upper-level ecology lab. Along with Dr. Larry Blumer from Morehouse College, he has developed the bean beetle as a model system for inquiry-based teaching in undergraduate biology laboratory courses.

Appendix A
DNA Extraction for MiSeq Analysis

Below we describe a phenol:chloroform extraction protocol. Stopping points are marked should the process take longer than one class period.

Materials needed:
- Sample to extract in a 1.5 ml tube
- 1.5 ml microcentrifuge tubes
- Sterile disposable pestle
- Liquid nitrogen
- Long forceps
- Centrifuge capable of reaching 15000 rpm and temperature controled
- Pipettes capable of volumes between 20-500 ul
- Fume hood
- Heat block or water bath

Reagents:
- CTAB
- 10% SDS
- Phenol: chloroform: isoamyl alcohol
- Chloroform
- 3M NaOAc
- Cold 99.5% ethanol (stored in -20˚C)
- Cold 75% ethanol (stored in -20˚C)
- Molecular grade water

The following steps are on the lab bench, no fume hood needed.

1. Using long forceps, dip the 1.5 ml tube containing the samples into a liquid nitrogen safe container.

2. Once sample is fully frozen, with a sterile pestle, crush bean beetle sample thoroughly.

3. Add 200 µL CTAB to the sample and continue crushing with the pestle. The same pestle can be used as long as it has not touched any other surface. (**Possible stopping point:** the next steps includes the bulk of the protocol, and the next stopping point is closer to the end of the protocol. Depending on time constraints, steps up to this point can be done on a different day and stored in the -20˚C freezer.) Mix by vortexing for 2 seconds. Incubate sample at 60˚C for 1 hour on a heat block or water bath (if heat block can shake, set to 400 rpm).

4. Add 40 µL of 10% SDS and mix by vortexing for 2 seconds. Place back on heat block and incubate for 1 hour at 60˚C.

Note: The following steps use phenol and flammable solvents. Work **must** be done in a fume hood and carefully supervised. When working with phenol, students should wear necessary protective equipment. Instructor must consult safety information on the usage and proper disposal of a materials that come in contact with phenol. Phenol has a very strong odor, if any student smells the phenol, tell the instructor immediately.

5. Add an equal volume (should be around 250 µL) of phenol: chloroform: isoamyl alcohol to the sample. The phenol reagent easily drips from the pipette, hold the tube close to the reagents container to prevent loss of volume and spilling.

6. Centrifuge at 15,000 rpm for 10 minutes. This will separate the top aqueous layer which contains the DNA, from the middle interphase and bottom organic layer which contains cell debris. While waiting for the centrifuge, label a new set of 1.5 mL tubes for each sample.

7. With a 200 μL pipette carefully transfer the upper aqueous layer and place in a new 1.5 mL tube. It is not necessary to collect all of the top layer, collect only what is comfortable without disturbing the interphase. Disturbing the interphase will add contaminants to the DNA which can affect amplification and sequencing. Discard the bottom organic layer.

8. It is possible that a small amount phenol remains in the aqueous layer after centrifugation. Phenol contaminants can prevent proper amplification and sequencing of the DNA samples. To remove possible phenol contamination, add an equal volume of chloroform (around 200 μL) to the transferred aqueous layer. Chloroform easily drips from the pipette as well, so keep the tube close to the chloroform container.

9. Centrifuge at 15000 rpm for 5 minutes. This will again separate the top aqueous layer which contains the DNA, from the middle interphase and bottom organic layer which will contain any remaining cell debris and phenol contaminant. While waiting for the centrifuge, label a new set of 1.5 mL tubes for each sample. Note that this this new tube is where the DNA will end up, therefore make sure to label properly.

10. Carefully transfer aqueous layer to the new labeled tube.

Note: The following steps can now be performed outside of the fume hood.

11. Currently the DNA is in solution and a pellet will not form. To precipitate DNA, add 0.1 volumes of 3 M NaOAc (around 20 ul) and 2 volumes of 99.5% molecular grade cold ethanol (around 400 μL, stored at -20˚C freezer). Gently mix the tube by rocking back and forth. Do not mix using the vortex for it might damage the DNA. Allow to precipitate for an at least 1 hour in the -20˚C freezer. **Possible stopping point**: Samples can also be left overnight in the freezer and work may be resumed on the next class day.

12. Centrifuge the tubes for 10 minutes at 15,000 rpm and at 4˚C.

13. A small white/clear pellet will form at the bottom of the tube, this is your DNA. Remove supernatant by carefully pouring it out. It is possible that a pellet will not be visible, however the DNA should still be adhered to the bottom of the tube.

14. Add 500 μL 75% cold ethanol taken directly from the freezer. Centrifuge at 15,000 rpm and 4˚C for 10 minutes.

15. Carefully pour out the supernatant as above. Gently and slowly pipette out any remaining supernatant without disturbing the pellet or the area where the pellet should be.

16. Allow the pellet to air dry by placing the tube sideways on the sterile bench top with the lid open for 5 minutes.

17. Resuspend the dried pellet in 30 μL of molecular grade water. Flick/agitate the tube. DNA can be left out at room temperature for the remainder of the class to help the pellet go into suspension. DNA can be stored in a 4˚C fridge for a few days or in the -20˚C freezer for long term storage.

18. Before sending the DNA off for sequencing, we recommend checking the DNA extraction was successful by using a nanodrop and by PCR.

Appendix B
Pre-lab Assignment on Bacterial Identification using 16S rRNA Sequence Data

Below is a sequence of the 16S rRNA gene from an unknown bacterial colony. Use the NCBI BLAST search and the pdf on computational tools to identify the species.

>UnknownBacteriaSpecies

NNNNNNNNNNNNNNNNNNNNNNNNNNNANNNTGCAAGTCGAGCGGTANCACAGAGAGCTTGCTCTCGGGTGAC
GAGCGGCGGACGGNNNNNNAANGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTA
ATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGAT
TAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCGTTAAGG
TTAATAACCTTGGCGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAA
ATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACNAAGACTGA
CGCTTCANGTGNCGAANNCGTGGGGNAN

1. Are there any hits returned from your BLAST search that cover the entire query sequence?

2. What are the most significant e-value and the associated query coverage from your search?

Resubmit your query using the sample sequence below. All of the leading N nucleotides (N = G, C, A, or T) have been removed.

>UnknownBacteriaSpeciesNoN

ATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAG
TAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCGTTAAGGTTAATAA
CCTTGGCGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCG
GGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTT

3. Are there any hits returned from your BLAST search that cover the entire query sequence? Why do you think the change in sequence affected the coverage?

4. What are the most significant e-value and the associated query coverage from your search?

5. Provide the genus and species of the unknown bacteria.

6. Use the Taxonomy report to identity the Kingdom, Class and Family of the bacterial species.

7. What other species were identified in the BLAST search?

8. Why might these hits be so similar to your sequence?

Appendix C
Google Form used to Collect Morphological Diversity Data

1. What is your name (needed for instructor to enter your points for completion of the survey)?
2. What sex was your beetle?
    a. Female
    b. Male
3. What type of bean was your beetle raised on?
    a. Adzuki bean
    b. Black eyed pea bean
    c. Mung bean
4. Species richness (number of unique colony types/species your group found based on morphology).
5. Inverse Simpson's Index (based on morphology data).

Appendix D
Google Form Used to Collect Sequencing Data

1. What is your name (needed for instructor to enter your points for completion of the survey)?
2. What sex was your beetle?
    a. Female
    b. Male
3. What type of bean was your beetle raised on?
    a. Adzuki bean
    b. Black eyed pea bean
    c. Mung bean
4. What type of plate was your colony grown on?
    a. EMB
    b. Blood agar
    c. Nutrient agar
    d. PEA
5. Colony size
    a. Small
    b. Medium
    c. Large
6. Colony color
    a. White
    b. Off-white
    c. Yellow
    d. Red
    e. Orange
    f. Brown
    g. Other
7. Colony gloss
    a. Shiny
    b. Matte
8. Colony form
    a. Circular
    b. Filamentous
    c. Irregular
    d. Rhizoid
9. Colony elevation
    a. Raised
    b. Flat
    c. Convex
    d. Crateriform
    e. Umbonate
10. Raw sequence.
11. Base number for start of trimmed sequence.
12. Base number for end of trimmed sequence.
13. Identified genus (or NA if could not identify genus).
14. Identified species (or NA if could not identify species).

Mission, Review Process & Disclaimer

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