THE EFFECTS OF CAPTIVITY ON THE ENDANGERED COMAL SPRINGS RIFFLE BEETLE, *HETERELMIS COMALENSIS*

by

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DEDICATION

To my Father who has been an inspiration and example by never letting go of his dreams. He and my mother have made untold sacrifices which have been paramount to my growth in college and essential to my success moving forward.

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LIST OF ABBREVIATIONS

Abbreviation	Description
CSRB	Comal Springs riffle beetle
EAA	Edwards Aquifer Authority
USFWS	United States Fish and Wildlife Service
SMARC	San Marcos Aquatic Research Center
WGS	Whole Genome Sequencing
RDP	Ribosomal Database Project
JGI	Joint Genome Institute
ASV	Amplicon Sequence Variant
LEfSE	Linear Discriminant Analysis Effect Size

ABSTRACT

Combining culture-dependent with culture-independent studies can offer insight into the ecological patterns between microbiomes and their environments. *H. comalensis*, commonly known as the Comal Springs riffle beetle (CSRB), is an aquatic, endangered species that feeds on biofilm from terrestrial detritus near the outflow of the Comal and San Marcos Springs. In this study, I characterize members of the CSRB microbiome by genetic and phenotypic methods. By combining these two methods, I can explain the ecological dynamics between the CSRB microbiome and its habitat in captivity versus the wild. Over 300 bacteria were isolated from beetles, water, and biofilm from beetle environments. After extracting the genome, I sequenced the 16S rRNA gene of 142 isolates to identify them to the genus-level. I found 30 genera belonging to 4 different phyla with culture methods and 22 genera within 10 phyla using high-throughput sequencing of the beetles. I found higher amounts of *Acidobacteria* and other phyla in the captive CSRBs from both culture and sequencing studies and confirmed the microbiomes were statistically different. A statistical analysis revealed 24 amplicon sequence variants (ASVs) that were significantly different between wild and captive beetles. Estimating the core microbiome also demonstrated that the beetle microbiomes are different in size and composition. This work has provided a skeleton for more detailed research into the mechanistic details of the CSRB microbiome.

I. INTRODUCTION

Animal-microbial symbiosis

Recent estimates suggest the earliest animal phylum, *Porifera*, first emerged about 600 million years ago, just before the Cambrian explosion, which also marks the time that animals began co-evolving with bacteria [1, 2]. Bacteria, which had already become metabolic masters of nearly every niche on the planet over the previous three billion years, have been symbionts to animals which contain consistent environmental and nutritional growing conditions. They may have contributed to early animal genomes by lateral gene transmission via bacterivory by more complex taxa[3]. One example of how bacterial genes may have infiltrated and influenced their hosts is with choanoflagellates, which consist of early animal relatives with a collar of microvilli capable of trapping bacteria that they can consume while exhibiting either a unicellular or multicellular morphology [3].

The hologenome theory, which proposes that hosts and their symbionts, the combination of which is referred to as a holobiont, act as distinctive biological entities due to the effects of the symbiont on host phenotype, heritability of the symbionts through vertical transmission, and exponential increase of genes, and therefore metabolic pathways, to the host [4]. This is one way bacteria enable animals to break down novel nutrients or traditionally nutrient-poor foods as they spread around the world [5, 6]. In return, animal digestive systems provide some of the most nutrient-rich places for bacteria to reside. The animal gut creates taxonomically diverse microbiomes which contain genes coding for chemical signaling, biosynthesis, and degradation pathways to which the host would otherwise not have access [7-9]. Microbiomes, which are the sum

of the microbes within a sample, are recognized as being critical to normal function of animals since they have relied on their microbes for millions of years.

Insect microbiome

Arthropoda is one of the largest and most diverse eukaryotic phyla with some scientists estimating *Coleoptera*, the beetles, to consist of approximately 25% of all described animal species [10]. Additionally, many insects contain some of the most diverse and unique microbiomes that are specialized for their specific environment and developmental stages [11]. Holometabolous insects, which are insects that progress through morphological changes as they develop, can gain and lose their gut microbiomes as they metamorphosize [12]. During development they are prone to stark changes as they advance through their instar stages [12, 13].

Many larval insects depend heavily on their microbiomes for normal growth and development [14-20]. One example is the mosquito (*Culicidae*), whose larvae will die if they do not receive bacteria that can create hypoxic conditions inside the larvae triggering development [14, 15, 18]. Mosquito larvae rely on specific gut bacteria received from the female parent to trigger chemical signals that advance instar stages making the bacteria essential for proper development. *Nicrophorus vespilloides*, a burying beetle which lays its eggs in fresh carrion relies on its microbiome, consisting of microbial-decomposing soil bacteria and fungi, to preserve carrion ensuring its larvae have fresh nutrients when they hatch and become colonized by the same microbes as they feed [16]. Morphological changes can take many shapes, but frequently the microbiome is purged during metamorphosis and insects must restart colonizing themselves with bacteria from their

new environment [12]. These instances of recolonization provide opportunities for major swings in microbiome composition making it important that the insect acquire the right bacteria to increase its chances for survival. Such requirements mean that insects that consume or live closely with biofilms are especially vulnerable while moving from different environments.

The endangered Comal springs riffle beetle

The Comal Springs riffle beetle (CSRB; *Heterelmis comalensis*) is a holometabolous endangered aquatic beetle that inhabits tight places near the outflows of the Comal and San Marcos springs in Central Texas [30, 31]. CSRB likely comes in contact with a wide variety of microbes from its diet of biofilm growing on terrestrial detritus that falls into the water as well as from its habitat near the riverbed [32, 33]. While originally it was believed that the CSRB pupated above the waterline, pupating CSRB have been found in between rocks and other tight places underwater [30]. Its small size of about 20mm makes it make it hard to identify without a microscope [34]. The beetles lack gills but use a collection of small hairs on the underside, referred to as a plastron, to trap air bubbles which they use to breathe [35]. It is also believed that the beetles can use the bubbles as a way of ascending and descending in the water [35].

Due to decreases in the quality and quantity of water from the Edward's Aquifer, which feeds the springs, caused by increased anthropogenic use and recent droughts, the Edwards Aquifer Authority (EAA) has contracted the United States Fish and Wildlife Service (USFWS) to build a refugium for the beetles [31, 36]. This refugium was designed to develop refuge populations that can be studied in the event of a drastic drop

in population size in the wild [37]. The USFWS has developed the refuge but have found the number of beetles reaching maturity within it is abnormally low due to low pupation rates and subsequent ecdysis and eclosion [30].

The effects of captivity on animal microbiomes

In captivity animals are often faced with standardized foods, medical treatments, and unvarying environments, which limit the diversity of their microbiome to the microbes they brought with them and the ones available in the captive environment [21]. For example, the effort to preserve the endangered Armargosa vole (*Microtus californicus scirpensis*) has been quite successful in terms of animal husbandry, however they fail to survive after they are released to the wild, likely due to the incompatibility of their microbiome with the new environment; captive vole microbiomes may have contributed to their inability to absorb enough nutrients in the wild after being released due to shifts in food availability [22]. To improve conservation efforts, sequencing the microbiome has been suggested for use as an estimation of success for captive-reared animals' survival in the wild because of the microbiome's contribution to physical ability [21]. Many wild microbiomes are estimated through fecal samples which are not always representative of the total gut microbiome. Standardized methods for establishing core microbiomes are needed, as well as improved wild-animal microbiome sampling to establish baselines for comparisons with captive animals and monitor microbial changes in animal populations over time [22].

Potential of functional analysis with cultured bacteria

Since the end of the 1980s, 16S rRNA sequencing and whole genome sequencing (WGS) of metagenomes are the methods of choice to analyze the structure and composition of microbial communities [23]. DNA sequencing is a destructive method that often makes the sequence unviable for future physiological studies of the microorganisms in the sample. Culture-dependent studies can help fill this gap. Growing and isolating bacteria on agar plates is the traditional method used to study bacteria for many years. From these isolates it becomes possible to build frozen stock libraries by preserving the bacteria in a glycerol solution for later studies and, therefore preserving the culturable diversity of the sample. The Great Plate Anomaly is the observation that only 1% of the bacteria that current techniques are able to culture actually make up the diversity of bacteria that are present in the sample [24]. This speaks to the importance of continuing to develop new methods of growing bacteria for culture studies. These studies provide valuable information that can be paired with genome information to find novel gene clusters and discover new pathways for a deeper understanding on how microbes interact with their hosts to increase their survivability [25, 26].

Goals

The composition of aquatic animals' microbiome is influenced by various environmental factors, such as, diet, climate, water quality and others. I hypothesize that if captive environment fails to mimic the conditions experienced by wild populations of *H. comalensis*, then the microbiome of wild and captive beetles will differ.

- Objective 1: Utilize culture-dependent methods to characterize and compare the microbiomes of wild and captive adult *H. comalensis*.
- Objective 2: Characterize and compare the microbiomes of wild and captive adult *H. comalensis* with high throughput 16S rRNA gene sequencing.

II. CULTURE-DEPENDENT STUDY OF CSRB MICROBIOME

Introduction

With the recent explosion of new sequencing technology many microbiologists have shifted focus from culture studies to newer technology. One reason they have moved away from biochemical tests and culturing microbes on agar plates is the increased amount of information and resolution genomic sequencing provides [27]. Sequencing the genome gives a higher level of identification for bacteria while also providing a complete list of all their potential functions. Massive repositories have been constructed to contain genetic information collected for comparing new genomes to known bacteria sequences. As the genes linked with new pathways are discovered and incorporated into databases, even greater clarity can be gained on how a bacterium might function. However, sequencing the genome cannot replace biochemical tests because they determine with a high level of certainty that the bacteria is able to complete a certain job [28]. Sequencing genomes can only identify known pathways and not potentially novel ones. There are many gaps in bacterial genomes that sequencing does not yet fill demonstrating the need for both culturing and biochemical tests [29]. Pairing culture-dependent with the culture-independent information can provide insight to the function of bacteria within controlled environments [26].

Many different media types have been developed to imitate certain environments. One example is R2A, a media that has low amounts of nutrients and is designed to mimic freshwater environments. Other media, like BHI and nutrient agar, were developed to provide large amounts of different molecules by taking plant or animal products and using digestive enzymes to break down complex molecules into a variety of potential

carbon sources for a diverse assortment of bacteria. Unfortunately, culture techniques, like these, are only enough to culture approximately 1% of the bacteria that are present in a sample but are still valuable for finding bacterial strains that influence their environment and novel genes by combining gene sequencing and biochemical tests [30].

Insect microbiomes are diverse and since captive environments can have a detrimental effect on the diversity and makeup of insect microbiomes, it is possible that insects experiencing issues in captivity are missing bacteria with an essential function or are infected by potential pathogens [11]. To study potential pathogens, the bacteria should be isolated and introduced to a sterile insect to identify its mode of pathogenicity. Essential bacteria or essential genes can be individually introduced to axenic beetles to study the bacteria and its genes effects on pathways that the beetle needs for normal functioning and development [14, 15]. This chapter describes the isolation and identification of bacteria associated to *H. comalensis* and their habitat. This study will provide some groundwork for future studies into potential pathogens and beneficial bacteria that can modulate the health and development of insects by finding culturable bacteria that differs between wild and captive beetles. By preserving bacteria that could be cultured it preserves the culturable diversity of the beetles and their environments.

Materials and Methods

Bacterial isolation

The USFWS at the San Marcos Aquatic Research Center (SMARC) located in San Marcos, Texas collected 15 CSRBs for a culture-dependent study and delivered them to the lab at the Ingram School of Engineering in water. Of the 15 beetles brought for this

study, eight were wild beetles collected from Comal Springs and seven were from the refugium at SMARC. The beetles from the refugium at SMARC were collected from Comal Springs and given at least six months to adapt to the conditions before being used for sampling. The beetles were euthanized by refrigeration at 4℃. Each beetle was individually washed in sterile PBS solution with 100µL of wash plated on R2A, BHI, and nutrient agar. Each beetle was homogenized in a sterile microcentrifuge tube and pestle in 100µL of PBS, which was inoculated on R2A, BHI, and nutrient agar, and all bacteria were incubated at 26℃ for about two weeks. These plates were referred to as the source plates for each beetle because they contained the bacteria that were isolated for downstream applications.

In addition to the beetles, biofilm and water samples were also collected from the same locations by scraping the biofilms off the substrate with a sterile razor blade. The biofilm was collected from pieces of wood that were located near the collection site of the beetles as well as from the refugium. Each biofilm sample was dissolved in sterile PBS and inoculated on R2A, BHI, and nutrient agar and incubated at 26℃ for two weeks. The water source plates were made by plating 100µL of water directly onto R2A, BHI, and nutrient agar and grown at 26℃ for the same amount of time.

16S rRNA sequencing and analysis

The genomes of each isolate were extracted using the PureLink® Genomic DNA Mini Kit by Invitrogen. From the genomic DNA the 16S rRNA gene was amplified using the primers 27F (AGAGTTTGATCMTGGCTCAG) and proK1492R (GGWTACCTTGTTACGACTT). The success of each amplification was checked by

running the sample on a 1.5% agarose gel at 90V for 25-30 minutes. Each amplicon was purified using the GFX™ PCR and Gel Purification Kit by GE. Each set of 16S rRNA gene amplicons were quantified using the Qubit 4 fluorometer to ensure the minimum amount of 10ng of DNA required by the Center of Research Support at The University of Texas – Austin where the 16S rRNA genes were sequenced with Sanger sequencing. Any samples that exceeded 4ng/µL were diluted to 1 ng/µL by adding nuclease-free water to the sample prior to shipping. Once the sequence reads were available, each forward and reverse sequence was corrected in Chromas v.2.6.6 and reads with an average quality score below 30 were discarded. Contiguous sequences (contigs) were aligned from the corrected reads using UGENE v.1.3.7 and stored in a FASTA file. The FASTA file was uploaded to nBLAST, SILVA, and the Ribosomal Database Project (RDP) and taxonomically identified to the genus-level. Each identity was compared with results from other databases acting as a quality check for each other. The nBLAST results and their accession IDs were recorded and assigned as the likely genus-level identification of the isolate. The closest neighboring 16S rRNA gene sequence, according to the SILVA database, was added to a file of sequenced isolates and trimmed to make sequences added by SILVA the same length as the isolates in UGENE v.1.3.7 before calculating a phylogenetic tree. The phylogenetic tree was calculated in MEGA-X using a bootstrap method (500) for phylogeny test and the General Time Reversal substitution model. Partial deletion was allowed with a site cutoff of 95%. Nearest-Neighbor-Exchange was used for the ML heuristic method. Isolate genus and SID# were added to the tree clades with FigTree v.1.4.4 and additional illustrations added in Adobe Illustrator for clarity.

Results and Discussion

Initially, 329 isolates were grown across all the media and source types with 236 isolated from homogenized beetles and 93 from biofilm and water samples. Isolates from wild beetles totaled 81 while 155 isolates were from captive beetles. Wild wood biofilm samples produced 27 isolates and only two isolates from captive wood biofilm were successfully grown. Water samples collected from Comal springs contained 52 isolates while 12 isolates were grown from refugium water samples. While freezer stocks were prepared, 45 isolates were lost due to prolonged exposure at suboptimal temperatures, 18 were found to be contaminated or not fully isolated, and 54 isolates were unable to grow in liquid media. After lost or contaminated isolates were eliminated, 223 isolates were made into frozen stocks and stored in a culture collection at -80°C.

After forward and reverse sequence reads of the 16S rRNA gene were returned from the Center of Research Support, 81 contigs could not be formed due to low quality of either the forward or reverse reads. The FASTA file containing the 142 contigs was uploaded to nBLAST, SILVA, and RDP for identification and comparison across multiple databases. The dataset contained 34 genera with eight genera that were present in both wild and captive beetles. Samples from wild and captive water samples only had one isolated genus in common: *Acinetobacter*.

According to the cultured bacteria, the phylum *Proteobacteria* contained the largest number of isolates from both wild (47) and captive (50) sources. In sources from the wild the other phyla in descending order of abundance were *Bacteroidetes* (16), *Firmicutes* (5), and *Actinobacteria* (3). Isolates from captive sources were identified as being from the same phyla of *Firmicutes* (12), *Actinobacteria* (3), and *Bacteroidetes* (2).

Table 1: The number of beetles from each source. Beetles followed by the isolate totals and biofilm substrate from their respective source. Total values are the sum of the isolates from each source.

	Beetles	Beetle Isolates	Wood Isolates	Water Isolates	Total
Comal Springs	8	81	27	52	**160**
SMARC	7	155	2	12	**169**

Table 2: The number of isolates that were collected from Hc# (*Heterelmis comalensis*). Shaded region shows beetles that were collected from SMARC as well as the total isolate values. Average values are the average isolates per beetle from a source.

	Comal Springs								SMARC									Total
Beetle ID	Hc1	Hc2	Hc3	Hc4	Hc5	Hc6	Hc7	**Avg**	Hc8	Hc9	Hc10	Hc11	Hc12	Hc13	Hc14	Hc15	**Avg**	
# of Isolates	13	9	13	1	20	15	10	**11.6**	24	8	35	24	30	18	6	11	**19.5**	**237**

Table 3: The genera of isolates from beetles and their quantities. Number in parentheses is the number of beetles used.

	Beetle	
	Wild (7)	**Captive (8)**
Acidovorax	1	0
Acinetobacter	2	4
Aeromonas	6	1
Bacillus	3	4
Brevundimonas	1	5
Chromobacterium	0	2
Chryseobacterium	3	1
Comamonas	0	3
Dunganella	0	1
Enterococcus	1	0
Gordonia	0	1
Herbaspirillum	0	1
Mangrovibacter	0	1
Massilia	0	1
Mycobacterium	0	1
Novoaphingobium	0	1
Pelomonas	0	1
Pseudomonas	2	3
Rhizobium	0	1
Roseateles	1	0
Sphingomonas	0	1
Sporosarcina	0	1
Staphylococcus	1	2
Stenotrophomonas	0	1
Tsukamurella	2	3
Unclassified	0	1
Total	**23**	**41**

Table 4: The genera of isolates from water and their quantities. Number in parenthesis is the number of samples that were plated.

	Water	
Genus	**Wild (7)**	**SMARC (1)**
Acidovorax	0	1
Acinetobacter	1	1
Aeromonas	3	0
Arcicella	1	0
Bacillus	1	0
Brevundimonas	0	1
Chitinimonas	1	0
Chromobacterium	0	1
Chryseobacterium	2	0
Comamonas	1	0
Flavobacterium	1	0
Mangrovibacter	1	0
Pelomonas	0	1
Pseudomonas	2	0
Streptomyces	1	0
Total	**15**	**5**

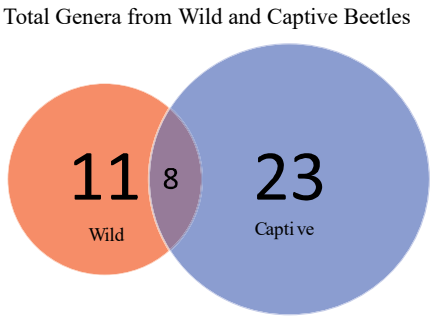

Figure 1: Venn diagram numbers indicate the total number of different genera from wild and captive beetles or genera that are present in both.

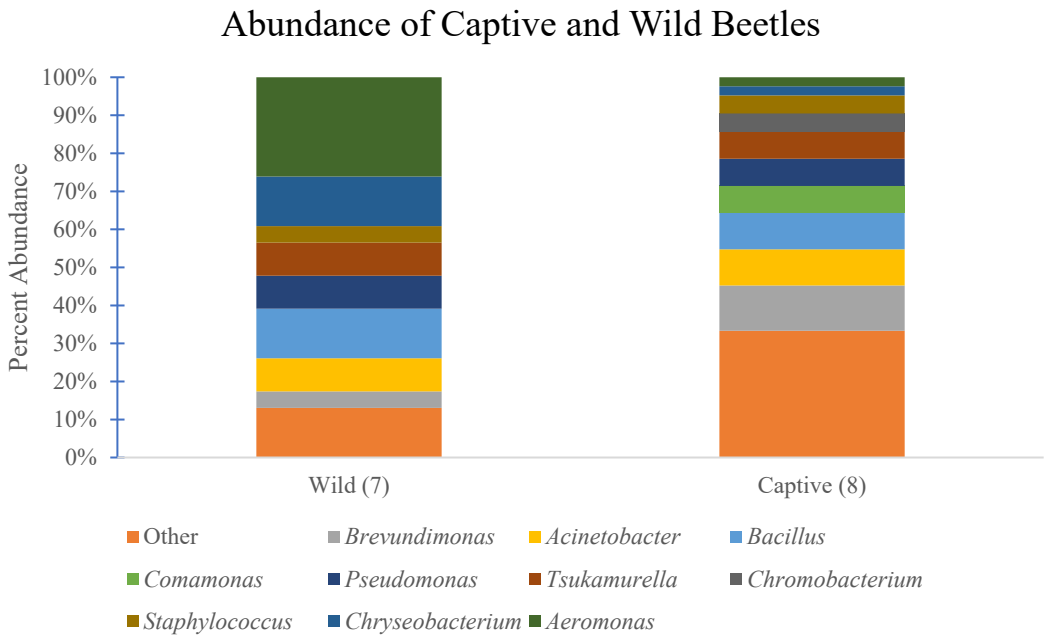

Figure 2: Relative abundance of culturable isolates at the genus-level from both wild and captive beetles. Genera with only one isolate are grouped into the 'other' category. The number in parentheses indicated the number of beetles from wild and captive sources used in the analysis of the 16S rRNA gene analysis.

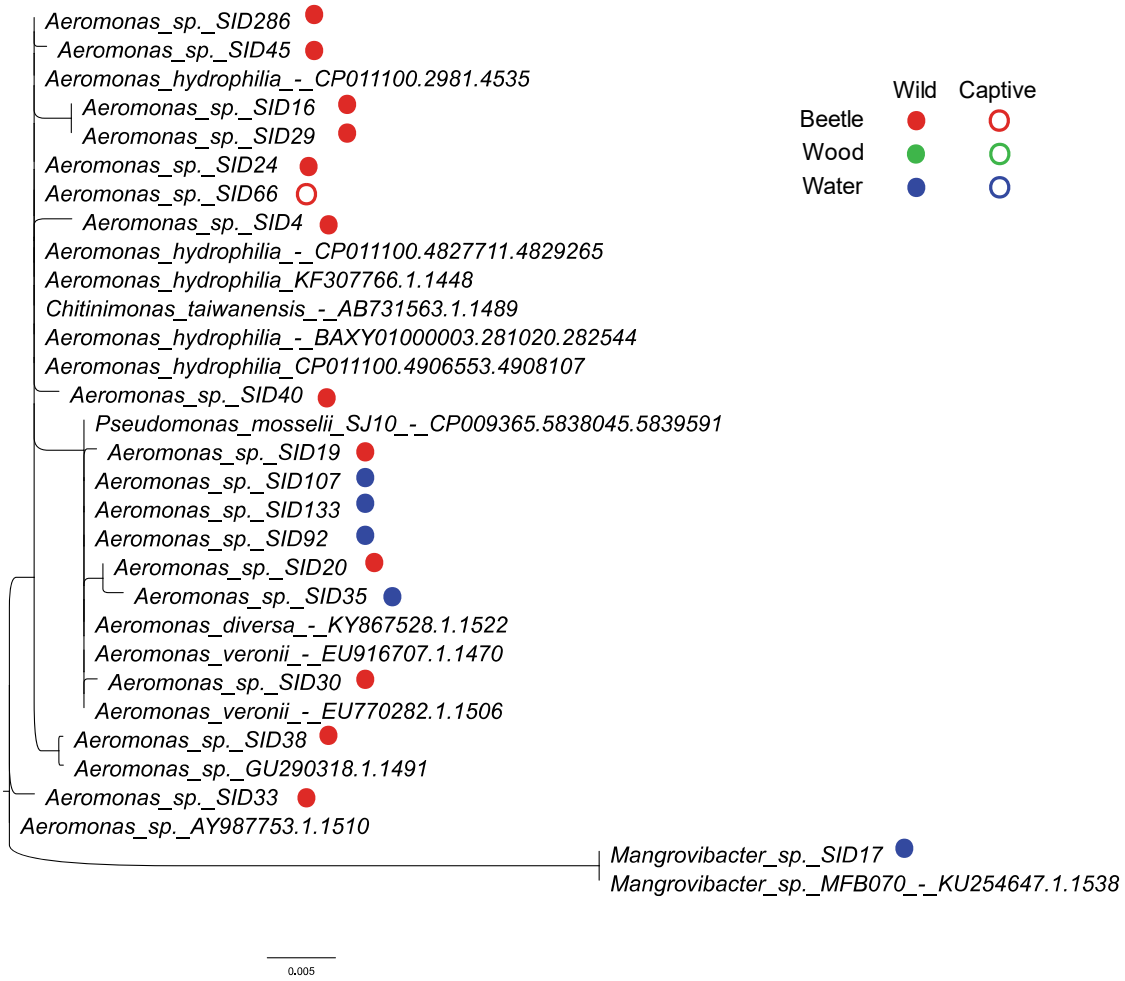

Figure 3: Phylogenetic tree containing the isolates within the order of *Enterobacterales* and *Aeromonadales*. It is made with database neighbors of 18 isolates with sequences 16S rRNA genes from the SILVA database. The tree was produced with a bootstrap method for phylogeny test and the General Time Reversal substitution model. Partial deletion was allowed with a site cutoff of 95%. Nearest-Neighbor-Exchange was used for the ML heuristic method. Dot color represents the type of substrate that the isolate was found, and the number of dots represents the whether the isolate came from wild or captive sources. Isolates without dots are neighboring sequences from the SILVA database.

Figure 4: Phylogenetic tree containing the isolates within the order *Pseudomonadales* and their closest neighbors from the SILVA database. The tree was produced with a bootstrap method for phylogeny test and the General Time Reversal substitution model. Partial deletion was allowed with a site cutoff of 95%. Nearest-Neighbor-Exchange was used for the ML heuristic method. The number of dots is representative of the sampling location and the color represents the substrate. Isolates without dots are neighboring sequences from the SILVA database.

Fewer bacteria were cultured from biofilms than adult beetles, which might be attributed to detection and inclusion of the whole beetle instead of only the digestive tracts where bacteria from biofilm the beetles might be ingesting would reside (**Table 1**). The biofilm bacterial community may not be as diverse as the microbiome in the beetles and may only account for a small percentage of the CSRB microbiome despite being a major component of their diet. Even though fewer wild beetles were used for culturable

bacteria diversity, this did not contribute to the low amounts of culturable wild beetle bacteria. The average cultures per beetles were much less than the captive beetle cultures per CSRB (**Table 2**).

By sequencing the 16S rRNA gene of the isolates it became apparent that the diversity of culturable bacteria was greater in captive beetles than in wild ones (**Table 3, Figure 1**). However, the diversity of water samples was reversed with wild water samples containing more culturable diversity than the water samples from the refugium (**Table 4**). Even though source water from the refugium also comes from the Edward's Aquifer, the location and environments surrounding the water source are different. The refugium pumps water from the aquifer in San Marcos, Texas while the collected water near wild beetles comes from surface water in New Braunfels, Texas. Surface water near spring outflows and water in aquifers can have different microbiomes because of the differences in water composition [31, 32]. This might account for the differences in culturable bacteria in water samples. It might also influence the bacteria available to the beetles in the refugium.

While the captive beetles had a higher culturable species diversity, they contained more rare species than the wild beetles (**Figure 2**). Selective pressures in the wild environment are likely greater because of possible runoff from the city of New Braunfels that surrounds it, changes in climate, or differences in trophic levels [32]. However, since we are only looking at the culturable bacteria in this chapter, which only make up about 1% of the bacteria that are present, no conclusions can be drawn on the basis on abundance values. Even though only a small number of bacteria can be tested with biochemical tests, it is not an excuse to avoid trying altogether. Culturable A*cinetobacter*

species are present in every tested source (**Figure 4**) but appeared in greater abundance in the captive CRSB gut microbiome. This has been linked to bacterial infections in other insects [33, 34]. *Aeromonas* appeared almost exclusively in wild beetle and water samples which could also be a source of infection (**Figure 3**). Additionally, a strain of *Acidovorax* was able to be cultured from the SMARC environment, which is present in large amounts according to the gene study.

Culturing these bacteria is a necessary first step to sequencing their genomes to look for necessary gene pathways in normal functioning beetles that might be filled by bacterial genes. Preserving the culturable diversity of the beetles and the beetle environments from both sources is also important for future studies where single strains may be inoculated into beetles to observe the effects of bacterial genes on beetle function.

III. CULTURE-INDEPENDENT STUDY OF CSRB MICROBIOME

Introduction

Microbiomes, the complete collection of microbes within a sample, have been established as contributors to the overall health of plants, animals, and their environments [35-37]. Since each microbiome is unique to the environmental pressures and available microbes of its location, identifying the health impacts on their hosts can be difficult to measure [38]. However, increased affordability and advancements in next generation sequencing technologies has made sequencing microbiomes available for projects with decreased funding [39]. Metagenomic analysis is the current mainstream method used to estimate which microbes are present and in what quantities by sequencing all the available DNA in a sample. Methods targeting the 16S rRNA gene of bacteria can sidestep host DNA for a look at bacteria exclusively [40]. Since some bacteria have multiple copies of the 16S rRNA gene and other rare species may not get amplified for several PCR cycles, estimating abundance accurately is challenging [41]. However, comparing several samples within a single treatment can illuminate trends in the data and be useful when comparing two treatments [42]. Some diversity metrics also account for excluded rare species while others weigh richness to estimate diversity making it important to include several indices for a more accurate picture of the data.

Microbiomes have caught the attention of conservationists as one way to measure a captive animal's ability to successfully rejoin the wild [43, 44]. Proximity with other animals, controlled diets, and disease are some examples of conditions that can alter gut microbiomes away from what is needed to live a healthy life [45]. However, estimating a healthy gut microbiome as a baseline can help determine how a captive animal will

perform after being released by comparing the baseline with the captive animal's gut microbiome [45]. A baseline can also be useful when adjusting a refugium for endangered species to ensure that they have the capacity to gain the nutrients they need [36]. Incorporating these measures with conservation efforts may increase the survivorship of captive animal released to the wild [46].

Insects contain some of the most diverse microbiomes and are prone to stark changes as they advance through their instar stages [12, 13]. Many larval insects depend heavily on their microbiomes for normal growth [14-20]. One example is the mosquito (*Culicidae*), whose larvae will die if they do not receive bacteria that can create hypoxic conditions inside the larvae triggering development [14, 15, 18]. Holometabolous insects, which are insects that progress through morphological changes as they develop, can gain, and lose their gut microbiomes as they metamorphosize [12]. The gut microbiome of *Nicrophorus vespilloides*, a carrion beetle, preserves carrion for its larvae by preventing the normal decomposition of the environments surrounding the ovisac [16].

The San Marcos and Comal Springs are fed by the Edwards aquifer, which has been experiencing decreased volume and quality due to increased pumping and pollution [47]. As a result of the declining environmental conditionals of the Edward's Aquifer, the Edwards River Authority has contracted the USFWS to develop a refugium in the event of a drought causing the death of the CSRB. SMARC, which is operated by the USFWS, has been housing the refugia for these beetles since placing it on the endangered species list [48]. However, the beetles are experiencing lower than expected pupation rates and subsequent ecdysis and eclosion as adults [49]. Due to links between gut microbiome and developmental health, it is possible that the larvae in the refugium are experiencing

dysbiosis [15, 18]. Dysbiosis is the decrease in health caused by changes in the microbiome away from what is considered normal [35]. Studying these links to health has become possible with the rise of next generation sequencing and has spurred the new field of metagenomics [50, 51].

Using the V4 region of the 16S rRNA gene to taxonomically identify bacteria to the genus-level, we compared the microbiomes of adult CSRBs that had been living in the refugia and wild environments. Differences between the two microbiomes likely reflect differences in the water source microbiome since the beetles from the refugium are receiving water pumped from a different location in the Edwards Aquifer but similar detritus. Significant differences in the two microbiomes were found with a large difference in the captive beetles with the increased presence of the phylum *Acidobacteria*. Since the captive beetles already receive detritus from the spring outflow, it is possible that there is a difference in the water microbiomes that is causing dysbiosis in the CSRBs that may also be effecting developing larvae resulting in their premature deaths.

Materials and Methods

Sample Collection

SMARC provided 16 captive CSRBs from their refugium and 23 wild CSRBs from the outflows of the Comal Springs located in Central Texas. The captive beetles came from the January and May 2019 collections while the wild beetles were collected in May 2019 and during February of 2018. The wild beetles were collected using the cotton cloth lure method from Gibson et al. (2008) but with a blend of polyester and cotton to

prevent the cloth from degrading underwater. The captive beetles were reared with leaves and rocks from their original collection site in plastic bins for at least six months from the collection date at SMARC. Each bin had a constant flow of water from a source within the Edwards Aquifer near SMARC to simulate a natural flow of water from a spring outflow. Adult beetles were delivered as collections to the lab in >95% ethanol by SMARC staff and stored at 4°C until they were ready to be processed.

PCR amplification, amplicon sequencing, and data processing

Each sample contained one beetle since the number of beetles were limited due to its endangerment status. Each beetle was washed in 100µL PBS buffer by vortexing in a 1.5mL centrifuge tube. Using sterile mortars, the beetles were crushed before placement into a bead beater for 2 minutes at maximum speed. The metagenomes of each beetle were extracted from the homogenate using the Purelink Genomic DNA Kit by Invitrogen and stored in 50µL of elution buffer at -20°C. The DNA concentrations were estimated using the Qubit 4 fluorometer by Invitrogen. The forward and reverse V4 regions of the bacterial 16S rRNA gene in addition to barcode primers were amplified and cleaned with KAPA prior to sequencing with Illumina MiSeq using v2 chemistry.

Data Analysis

The analysis was performed using R software *v3.6.3*, which involved the formation of contiguous sequences about 250-300bp long, trimming primers and any nucleotides before the first nucleotide with a quality score of >2, and dereplication for filtering unique sequences with the *DADA2 v1.14.1* package pipeline [52]. Samples with

less than 1000 reads or drops in quality below 30 were removed from the analysis. Taxonomy was assigned using *silva_nr_v132_train_set.fa* to form the Amplicon Sequence Variant (ASV) table [53]. The ASV table was used for statistical analyses, including α-diversity, PERMANOVAs of groups, and estimating a core microbiome.

To check that the two groups of beetle microbiomes are significantly different, several statistical tests and graphical renderings were performed using the *MicrobiomeAnalyst* web platform [54, 55]. Alpha diversity of the beetle groups was estimated using the Simpson, Shannon, and Chao1 indices. The Chao1 index weights rare species more heavily and uses it to estimate the number of other rare species that may not exist in the sample. The Shannon diversity index also accounts for species richness and the number of rare species when estimating diversity. The Simpson index uses relative abundance and richness, but all three indices are considered standard and have their strengths and weaknesses. The relationships between wild and captive environments was plotted with a Nonmetric multidimensional scaling (nMDS) plot that was calculated using the Bray-Curtis Index. A PERMANOVA calculated the significance between the spread of the two groups and the differences of the samples within each group.

Results and Discussion

SMARC provided 16 CSRBs from Comal Springs (wild) and 23 from their on-site refugium (captive) for a total of 39 beetles. After checking the quality of the 16S rRNA reads and trimming sequences, four wild and two captive beetles were removed from the dataset leaving 12 captive beetle and 21 wild microbiomes that were used for the analysis. After cluster analysis of 269250 sequences, a total of 250 ASVs were found

in relation to the beetle microbiome. The phylum with the greatest difference in abundance was *Acidobacteria* that made up 17% of the sequences from captive and only 0.6% of sequences from wild beetles (**Figure 1**).

The Choa1 diversity metric estimates richness of captive beetles to be between 22 and 33 ASVs while the wild beetle richness is much lower between 12 and 20 ASVs. The wild beetle error bars overlap with the captive estimates and a few outlying wild beetles are considered to have greater richness than several captive beetles (**Figure 2**). The Simpson diversity index indicated that many of the beetles in the captive group have low diversity ranging from 0.82 to 0.94 while the wild group has a distribution of diversity among samples ranging from 0.7 to 0.95. However, the Shannon index indicated that the captive beetles had higher species richness and evenness, which is also demonstrated in the species abundance bar plot. The differences among captive beetles was smaller than the differences among wild beetles (PERMANOVA: F-statistic = 6.0023, $R^2 = 0.16222$, p-value < 0.001) (**Figure 3**). An ANOSIM using the Bray-Curtis distance metric was 0.4434 with a significance of 0.001. The *betadisper* function calculated the average distance to the median of the wild and captive beetles to be 0.5062 and 0.4330, respectively. A dendrogram of the samples show that there are major differences between most samples within the two groups, but three wild beetle microbiomes are similar to the captive beetles. Using the Linear Discriminant Analysis Effect Size (LEfSE) tool, 24 ASVs had abundances that were significantly different between groups (**Figure 4**). Wild beetle microbiomes contained six ASVs that were present in significantly great abundances than in captive beetle microbiomes and 18 were present in greater numbers in captive beetle microbiomes than in wild ones. The core microbiome of both the wild and

captive beetles was estimated by including ASVs that were present in 80% or more of beetles (**Figure 5**). The wild beetle microbiome contained two ASVs that were also present in the captive beetle core microbiome.

Table 5: Table of the calculated LEfSe algorithm results which is designed for biomarker discovery and metagenomics data. Kruskal-Wallis rank sum test detects significantly different features which is followed by a Linear Discriminant Analysis to calculate effect size or relevance.

ASV #	P values	FDR	Captive	Wild	LDA score	Phylum	Class	*Order*	*Family*	*Genus*
ASV024	6.33E-08	3.67E-06	332570	0	-5.22	*Acidobacteria*	*Blastocatellia_(Subgroup_4)*	*44159*	*NA*	*NA*
ASV008	4.00E-07	0.000011588	1280200	0	-5.81	*Acidobacteria*	*Blastocatellia_(Subgroup_4)*	*Pyrinomonadales*	*Pyrinomonadaceae*	*RB41*
ASV019	9.88E-07	0.000019107	323910	1543.1	-5.21	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
ASV062	0.000011758	0.00017049	100000	0	-4.7	*Proteobacteria*	*Gammaproteobacteria*	*Methylococcales*	*Methylomonaceae*	*Methyloglobulus*
ASV072	0.00019152	0.0022217	97035	973.8	-4.68	*Proteobacteria*	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	*Thiothrix*
ASV006	0.00040215	0.0035186	893500	255060	-5.5	*Bacteroidetes*	*Bacteroidia*	*Chitinophagales*	*Saprospiraceae*	*NA*
ASV004	0.00042465	0.0035186	1291700	194610	-5.74	*Firmicutes*	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*
ASV002	0.00059851	0.0043392	535470	2090900	5.89	*Firmicutes*	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*ZOR0006*
ASV046	0.00098314	0.0063358	98951	0	-4.69	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
ASV029	0.0023381	0.013561	188350	36518	-4.88	*Deinococcus-Thermus*	*Deinococci*	*Thermales*	*Thermaceae*	*Meiothermus*
ASV031	0.0037365	0.016671	174820	0	-4.94	*Acidobacteria*	*Blastocatellia_(Subgroup_4)*	*44159*	*NA*	*NA*
ASV053	0.0037365	0.016671	103390	0	-4.71	*Planctomycetes*	*OM190*	*NA*	*NA*	*NA*
ASV076	0.0037365	0.016671	46339	0	-4.36	*Bacteroidetes*	*Bacteroidia*	*Flavobacteriales*	*Weeksellaceae*	*Bergeyella*
ASV056	0.0079245	0.03283	0	42491	4.33	*Bacteroidetes*	*Bacteroidia*	*Chitinophagales*	*Saprospiraceae*	*NA*
ASV032	0.0095347	0.036868	55178	51936	-3.21	*Bacteroidetes*	*Bacteroidia*	*Flavobacteriales*	*Weeksellaceae*	*Apibacter*
ASV017	0.01184	0.040865	8874.3	177380	4.93	*Proteobacteria*	*Gammaproteobacteria*	*Betaproteobacteriales*	*Methylophilaceae*	*NA*
ASV074	0.01259	0.040865	91784	4443.4	-4.64	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Leptotrichiaceae*	*Sebaldella*
ASV009	0.012682	0.040865	589740	183310	-5.31	*Bacteroidetes*	*Bacteroidia*	*Cytophagales*	*Spirosomaceae*	*Emticicia*
ASV015	0.015559	0.047497	315010	113570	-5	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiales_Incertae_Sedis*	*NA*
ASV104	0.018583	0.053891	17982	1486.8	-3.92	*Bacteroidetes*	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*Niabella*
ASV007	0.020097	0.055505	99341	563070	5.37	*Bacteroidetes*	*Bacteroidia*	*Flavobacteriales*	*Weeksellaceae*	*NA*
ASV010	0.021713	0.057243	0	374430	5.27	*Proteobacteria*	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	*Thiothrix*
ASV040	0.029547	0.07451	97462	32266	-4.51	*Actinobacteria*	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	*Mycobacterium*
ASV052	0.034748	0.083974	0	135530	4.83	*Proteobacteria*	*Gammaproteobacteria*	*Betaproteobacteriales*	*NA*	*NA*

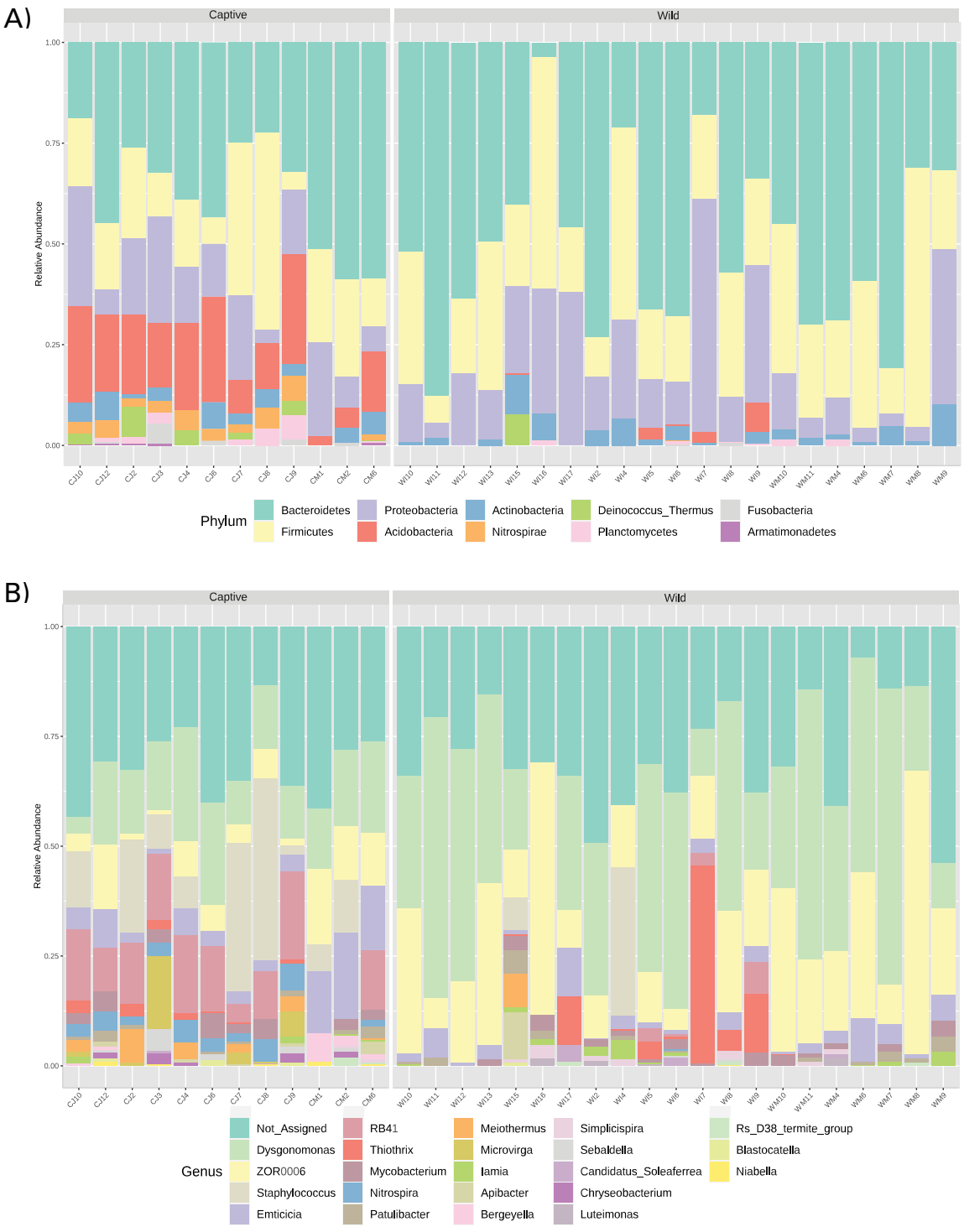

Figure 5: Relative abundance bar plots based on the (A) phyla and (B) genera of the beetle microbiomes. All taxa with counts less than 10 are merged for clarity. Bar plots are organized by source (Wild or Captive).

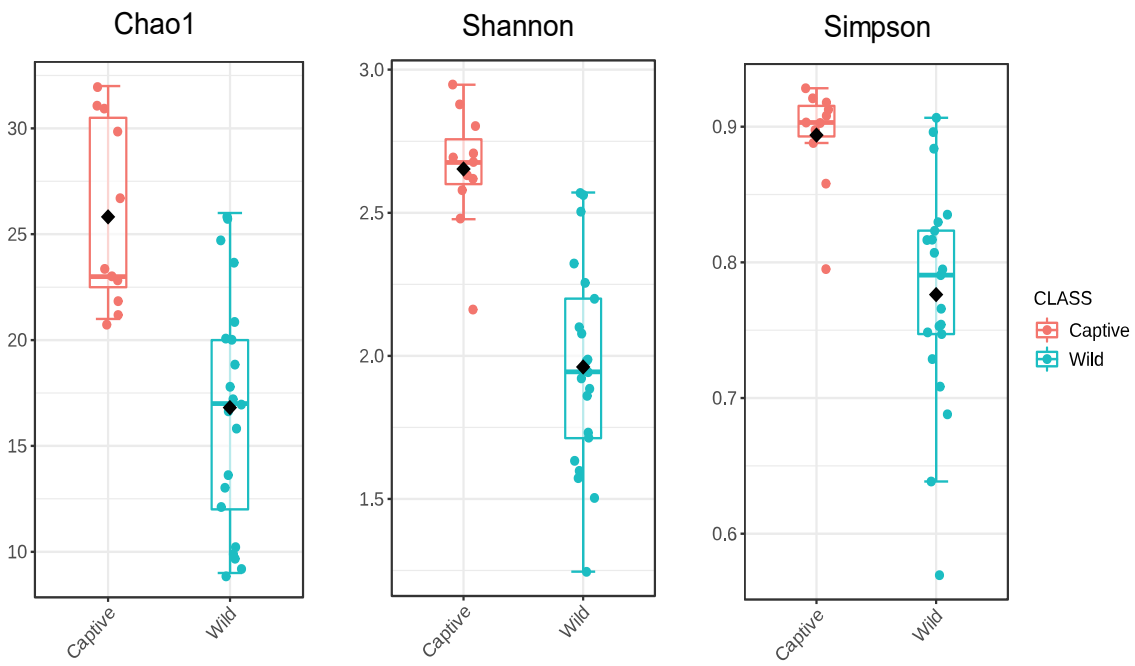

Figure 6: Chao1, Shannon, and Simpson diversity measures are used to calculate different estimates of diversity. The Chao1 method accounts for unobserved rare species that are indicated by the observed rare species. Shannon and Simpson diversity methods use species evenness and dominance in their estimation of diversity, respectively. Since the Simpson diversity index is less sensitive to low abundance taxa the Shannon diversity was also included to give a more robust diversity estimation of the two.

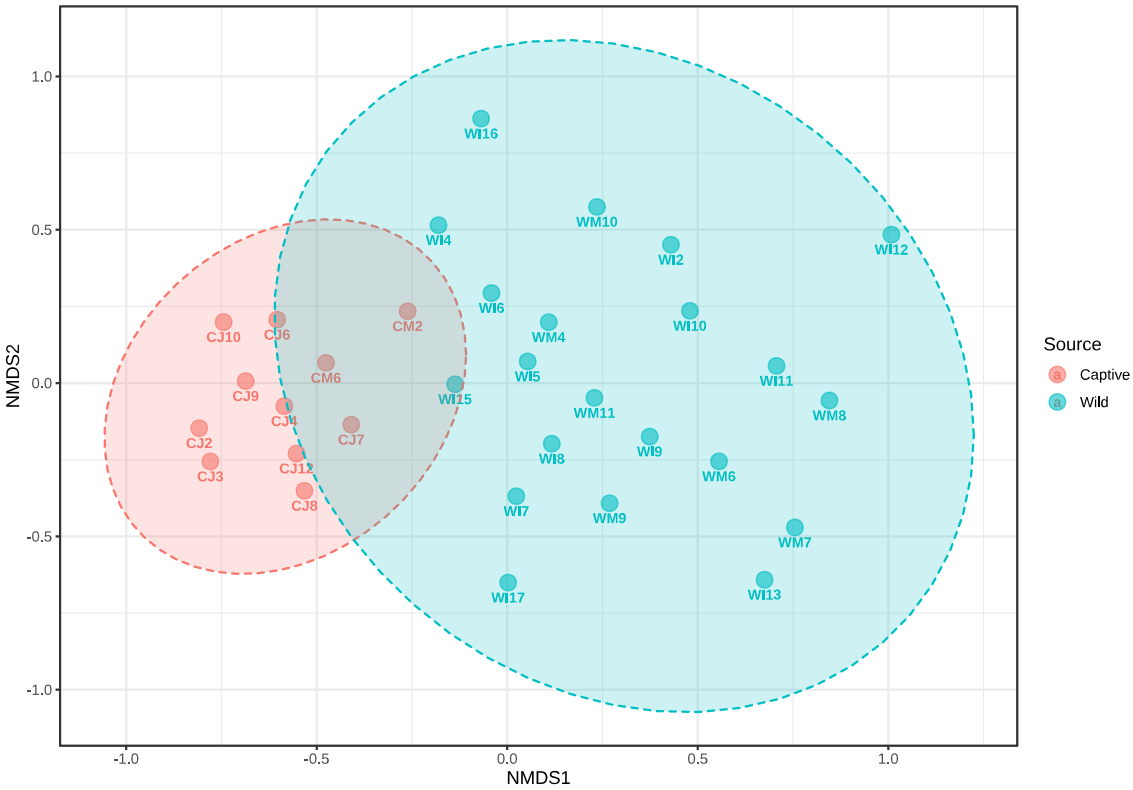

Figure 7: Non-metric multidimensional scaling plot which uses the Bray-Curtis Distance and Permutational MANOVA statistical method. F-value = 10.179, r-squared = 0.25334, p-value < 0.001, Stress = 0.18601.

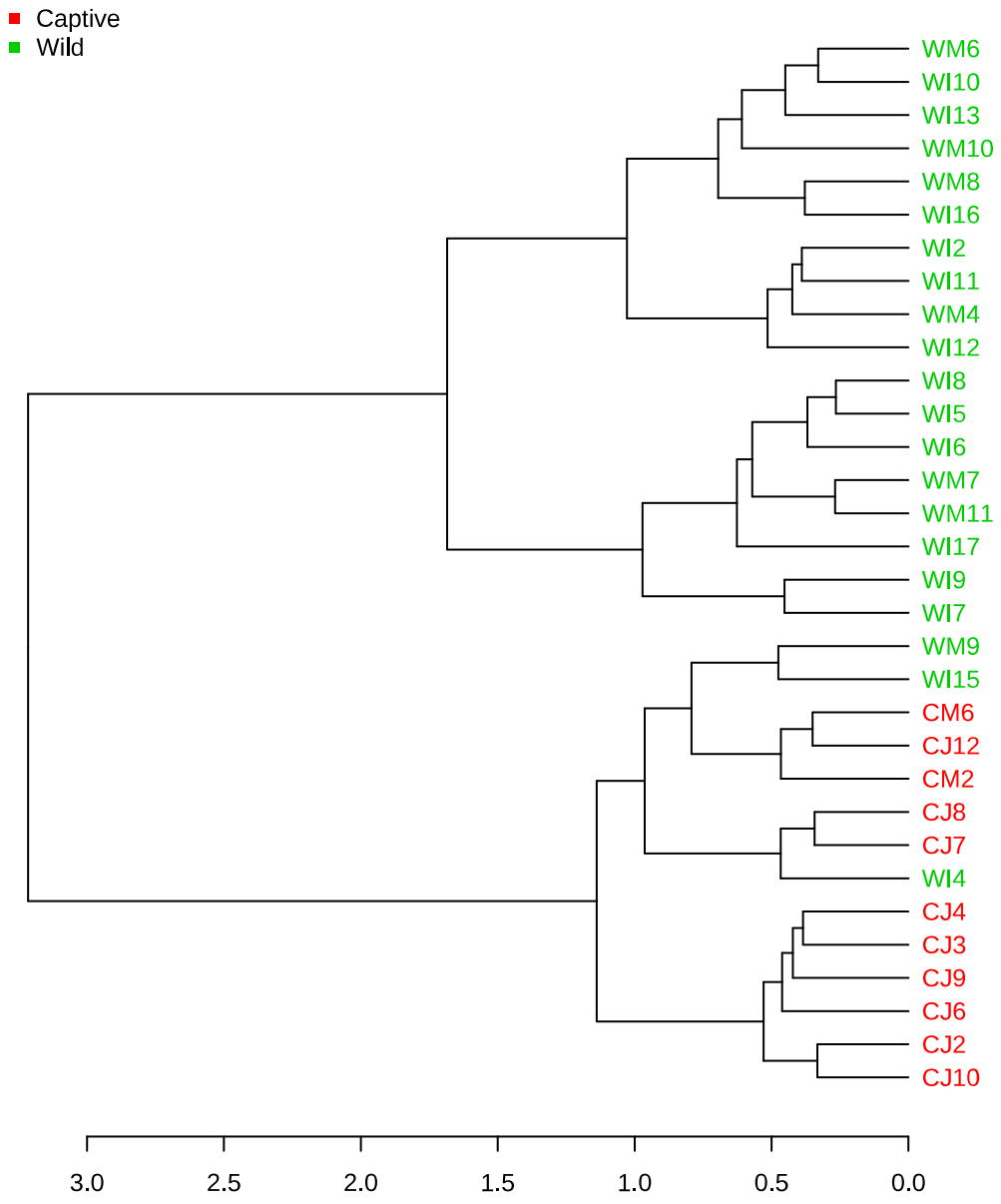

Figure 8: Dendrogram analysis of the wild and captive beetles formed using the Bray-Curtis distance measure and Ward clustering algorithm. Three wild beetle microbiomes were like the captive beetle microbiomes but overall, the two microbiomes appear quite different.

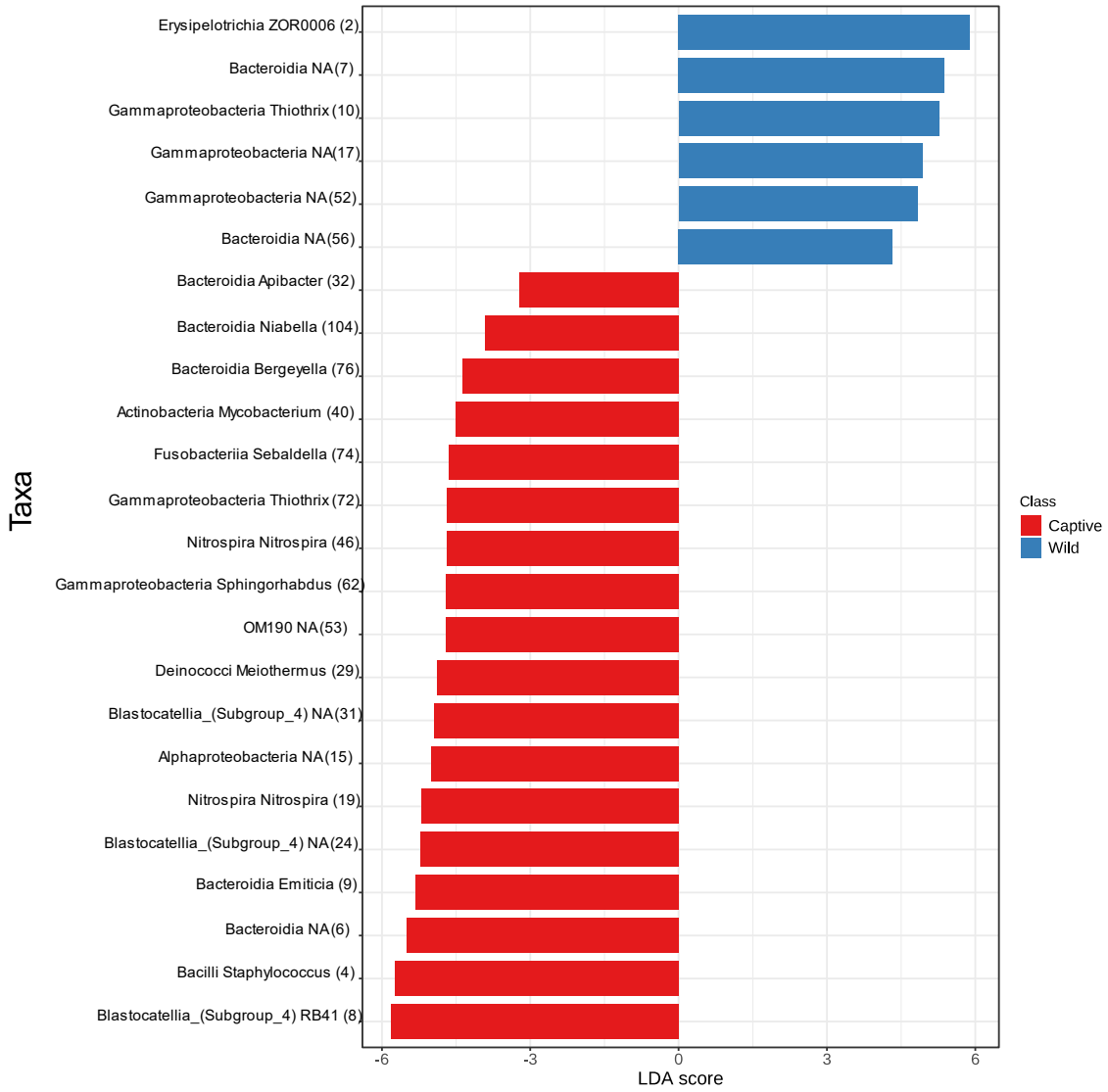

Figure 9: The Linear Discriminant Analysis (LDA) score shown as a bar plot. Linear Discriminant Analysis Effect Size (LEfSe) is a statistical method that shows which ASVs are likely to explain the differences between the captive and wild beetle microbiomes. P-value cutoff of 0.1 with an adjusted False Discovery Rate. Horizontal bars represent LDA score with a Log LDA score of 2.0. the number in parentheses represents the ASV that corresponds with the bar.

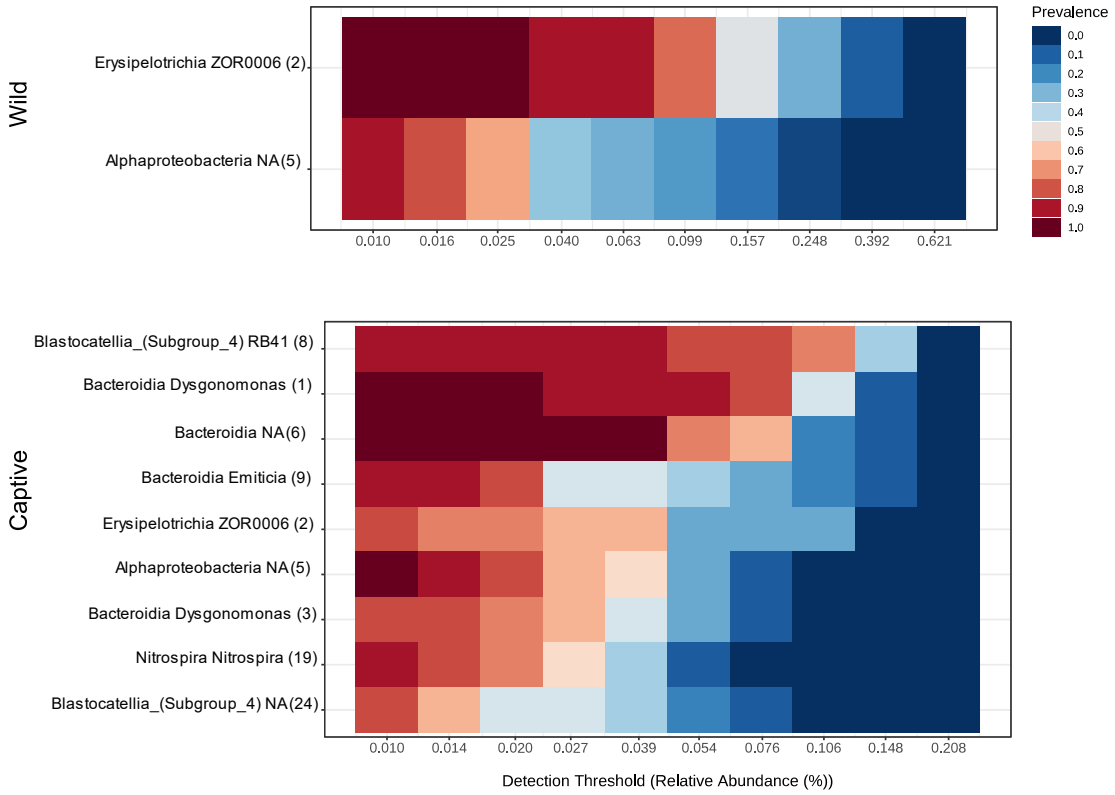

Figure 10: The core microbiomes of the wild and captive beetles were estimated based on a sample prevalence of 80%. The detection threshold is indicative of the sensitivity that the ASV is still able to be sensed. ASVs with a relative abundance below 0.01% were not included.

The major phyla in the wild beetles are *Bacteroidetes, Firmicutes,* and *Proteobacteria* while *Acidobacteria*, in red on the abundance bar plot, is a large component of the captive beetles that makes up only a small portion of the wild beetles (**Figure 1**). Captive microbiomes also contain more *Nitrospirae* and *Planctomycetes*, which occur rarely in their wild counterparts. *Nitrospirae* is a major phylum in nitrification but contains very few characterized bacteria species. At the (phylum) genus-level captive beetles contain more (*Acidobacteria*) *RB41* and (*Firmicutes*) S*taphylococcus* but the wild beetles contained more (*Bacteroidetes*) D*ysgonomonas* and (*Firmicutes*) *ZOR0006* compared to the captive beetles (**Figure 1**). *Acidobacteria* is

ubiquitous in soil samples, but its presence in captive beetle samples is not known. Other genera that had different amounts between the samples are common microbiome samples, in the case of Dysgonomonas [56].

The alpha diversity of both wild and captive CSRBs were compared with the Chao1, Shannon, and Simpson indices (**Figure 2**). With the CSRB 16S rRNA data, the Simpson diversity index shows that the captive beetles have fewer dominant taxa than the wild beetles, which contain a few high abundance taxa and several low abundance taxa at the phylum and genus level. The other indices estimate that the wild beetles have less diversity than the captive beetles as well as fewer rare species that might not be included in the sample. This is likely because of the increased weight given to low abundance ASVs which are prevalent in the captive beetles.

Another way of comparing multiple communities is with a nMDS, which represents the communities in as few dimensions as possible. Using this scaling method, the data was able to be represented in two-dimensions and portrayed by the ovals in **Figure 3**. The larger area belonging to the wild beetle microbiomes demonstrates the greater diversity of microbiomes among wild beetles estimated by nMDS scaling. It shows that the differences among the wild beetles is greater than the captive beetles, which is likely caused by the diversity of available bacteria in the wild versus captive environment. The wild environment is surrounded by populated parks and neighborhoods and is susceptible to runoff which likely contribute to the increases of diversity in the wild environment. The wild environment is also influenced by falling detritus, which is provides new biofilm for wild beetles to eat. Much of the biofilm used in captive beetle

environments is made in house using detritus from the wild environment and is not susceptible to the same environmental factors as the wild environment.

The dendrogram is a similar analysis that shows the hierarchical relationship between populations of the beetle microbiome samples (**Figure 4**). The dendrogram clearly separate all but three wild samples from the captive samples. The three wild samples that are closely related to the captive samples are: WI4, WI15, and WM9.

Linear discriminant analysis effect size (LEfSe) is an analysis designed for the discovery of significant genomic features [57]. *MicrobiomeAnalyst* can check for biologically significant ASVs within our dataset and determine how significant an ASV is and for which source (**Figure 5**). We found 24 ASVs that were considered significant with 18 from captive beetles and six from wild ones. Significance was determined based on a 95% confidence interval. Since the ASVs were based on 16S rRNA gene sequencing they could not always be identified to high taxonomic levels with some ASVs only being identified to the family-level and one ASV identified to the class-level (**Table 1**). This means that some ASVs that were not able to be identified were from new strains that have not been characterized in the past.

Lastly, estimating the core microbiome is becoming a standard in microbiome studies, but no official parameters exist. When estimating the core microbiome of the beetles the ASV must appear in 80% of the samples to be considered part of the microbiome. The core microbiomes for the captive and wild beetles were separately calculated in addition to the core microbiome of the whole dataset (**Figure 6**). Since the captive beetle have greater diversity the threshold for detection is much lower when estimating its core microbiome. The captive core microbiome also contained more

members than the wild microbiome, but these findings could be explained by the lower number of sequence reads from the captive microbiomes and possibly extenuating common species.

Any dissection takes practice to master and CSRB dissections are no exception. Prior attempts were made to dissect the intestinal tract out of the beetles, but since there was a limit on the number of CSRBs due to their endangered status, a decision was made to extract bacterial DNA from the whole beetles and avoid ruined beetles altogether.

Different bacteria have different copy numbers of the 16S gene and it is possible that the sequenced microbiomes are skewed to portray greater abundance of these bacteria [41]. Sequencing the full genome of each bacteria would allow for the quantification of the number of 16S rDNA in each bacterial strain, but new culturing methods need to be developed to grow currently unculturable strains. Shotgun sequencing would be able to determine the number of 16S genes but would result in high amounts of beetle DNA and lost bacterial strains in low abundance. For the purposes of identification of the greatest number of bacteria, targeted sequencing was chosen to eliminate non-bacterial DNA while maintaining reasonable confidence in the results. However, functional based analysis or transcriptomics data might provide greater resolution of the interactions between the microbiome and the beetles.

The high abundance of *Acidobacteria* could be a contributing factor of low pupation rates in the beetles, although little is known about this phylum's relationship with insects making it difficult to draw conclusions. *Acidobacteria* is a phylum of common plant and soil symbionts that have a wide range of phenotypes [58]. There are studies on other beetle larvae that have shown that closed ecosystems can yield lower

abundances of microbiomes, which would explain the decreased abundance of other phyla such as *Bacteroidetes* and, in some cases, *Proteobacteria* and *Firmicutes* [59]. It is also possible that the captive beetles are infected with something other than bacteria. Fungal and eukaryotic parasites are common and can alter the gut microbiome of insects among different developmental stages potentially inhibiting their growth [33].

Since it is possible for the beetles to develop resident microbes during their development in wild environments, it is important to look at the microbiomes of developing beetles to eliminate bacteria that were brought into the refugium as resident microbes. A study of the larvae during their instar stages would also shed light on possible necessary changes in their microbiome that they cannot get from the refugium. Since other insects, such as mosquitoes, require biochemical signals provided by bacteria in their microbiome, it is possible that the CSRBs are missing components of their microbiome that they are unable to get from their habitat in the refugium [14, 15].

IV. CONCLUSIONS

Some species of *Acinetobacter*, such as *Baumannii*, have been linked with infections and gut dysbiosis in other animals but it is common in water and soil globally [34]. Other genera were found in higher abundance within captive beetles and the refugia that require further investigation, such as *RB41*, a genus belonging to *Acidobacteria* which is common in soil microbiomes as part of carbon cycling, and N*itrospira*, a genus from the phylum *Nitrospirae* with roles in nitrite oxidation, but is poorly understood within insect microbiomes [60]. While captive beetles appear to have greater species richness, this could represent a decrease in efficiency of bacteria to perform a function. Species richness in wild beetles may be lower because they interact with more bacteria from countless sources enabling only the most efficient bacteria to find their niche with the developing beetles.

It is evident that the microbiomes of wild and captive beetles are different, but this could be caused by several reasons. The bacteria available in the captive environment are likely different than those in the wild environment and may serve functions that could be filled by other bacteria with similar gene pathways. These pathways might be essential for CSRB development just like other insects must have specific internal conditions that only bacteria can create. Using WGS to study water samples coming into captive and wild CSRB environments could demonstrate the differences in functional genes of bacteria in both locations.

It is possible bacteria that are colonizing beetles in captivity may leave gaps in pathways that are necessary for development. Using WGS of CSRBs at different instar stages in the wild could serve as a bacterial baseline that the USFWS should strive to

provide pupating CSRBs to promote ideal growing conditions. Transcriptomics can also provide evidence for activation of essential genes and what conditions they create at a given time. These and other techniques will narrow the number of pathways that larval microbiomes require to further study each instar.

Future research to look for pathogenic bacteria that can interrupt pathways and push the microbiome in a direction that is not optimal for pupation is necessary to further characterize factors that make the wild and captive environments different. Further investigating the genomes of the ASVs with significantly different abundances from the culture-independent study and matching them to isolates from the culture-dependent study could provide a short-list of potential pathogenic or essential bacteria that are currently in the culture collection. However, since many of the bacteria have not been cultured yet, experimenting with new methods of culturing bacteria that were significantly different between populations would be required. New culturing methods would contribute unique bacteria to culture collections and novel genomes that can be deposited in public databases and assist in future research on treating dysbiosis.

LITERATURE CITED

1. Rix, L., et al., *Heterotrophy in the earliest gut: a single-cell view of heterotrophic carbon and nitrogen assimilation in sponge-microbe symbioses.* The ISME Journal, 2020. **14**(10): p. 2554-2567.

2. Simion, P., et al., *A Large and Consistent Phylogenomic Dataset Supports Sponges as the Sister Group to All Other Animals.* Current Biology, 2017. **27**(7): p. 958-967.

3. Alegado, R.A. and N. King, *Bacterial influences on animal origins.* Cold Spring Harbor perspectives in biology, 2014. **6**(11): p. a016162-a016162.

4. Rosenberg, E. and I. Zilber-Rosenberg, *Microbes Drive Evolution of Animals and Plants: the Hologenome Concept.* mBio, 2016. **7**(2): p. e01395-15.

5. McCutcheon, J.P., et al., *Convergent Evolution of Metabolic Roles in Bacterial Co-Symbionts of Insects.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(36): p. 15394.

6. Li, Z., et al., *Metagenomic Analysis of Genes Encoding Nutrient Cycling Pathways in the Microbiota of Deep-Sea and Shallow-Water Sponges.* Marine biotechnology (New York, N.Y.), 2016. **18**(6): p. 659-671.

7. O'Donnell, M.P., et al., *A neurotransmitter produced by gut bacteria modulates host sensory behaviour.* Nature: International weekly journal of science, 2020. **583**(7816): p. 415.

8. Tyagi, A., et al., *Shotgun metagenomics offers novel insights into taxonomic compositions, metabolic pathways and antibiotic resistance genes in fish gut microbiome.* Archives of Microbiology, 2019. **201**(3): p. 295-303.

9. Prasad, R.K., et al., *Study on cellulase (B-1,4-endoglucanase) activity of gut bacteria of Sitophilus oryzae in cellulosic waste biodegradation.* Bioresource Technology Reports, 2019. **7**.

10. Stork, N.E., et al., *New approaches narrow global species estimates for beetles, insects, and terrestrial arthropods.* Proceedings of the National Academy of Sciences, 2015. **112**(24): p. 7519-7523.

11. Raza, M.F., et al., *Tephritidae fruit fly gut microbiome diversity, function and potential for applications.* B Entomol Res, 2020: p. 1-15.

12. Hammer Tobin, J. and A. Moran Nancy, *Links between metamorphosis and symbiosis in holometabolous insects.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2019. **374**(1783): p. 20190068.

13. Krishnan, M., et al., *Insect gut microbiome – An unexploited reserve for biotechnological application.* Asian Pacific Journal of Tropical Biomedicine, 2014. **4**: p. S16-S21.

14. Coon, K.L., et al., *Bacteria-mediated hypoxia functions as a signal for mosquito development.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(27): p. E5362-E5369.

15. Coon, K.L., et al., *Mosquitoes rely on their gut microbiota for development.* Molecular Ecology, 2014. **23**(11): p. 2727-2739.

16. Shukla, S.P.s.i.m.d., et al., *Microbiome-assisted carrion preservation aids larval development in a burying beetle.* Proceedings of the National Academy of Sciences of the United States of America, 2018. **115**(44): p. 11274-11279.

17. Solomon, G.M., et al., *The microbiota of Drosophila suzukii influences the larval development of Drosophila melanogaster.* PeerJ, 2019. **7**: p. e8097.

18. Wang, X., et al., *Bacterial microbiota assemblage in Aedes albopictus mosquitoes and its impacts on larval development.* Mol Ecol, 2018. **27**(14): p. 2972-2985.

19. Yin, W. and D.E. Rozen, *Gut Microbiota Colonization and Transmission in the Burying Beetle Nicrophorus vespilloides throughout Development.* Applied & Environmental Microbiology, 2017. **83**(9): p. 1-13.

20. Zhao, C., et al., *The Developmental Stage Symbionts of the Pea Aphid-Feeding Chrysoperla sinica (Tjeder).* Frontiers in Microbiology, 2019. **10**(2454).

21. Wang, L., et al., *Père David's deer gut microbiome changes across captive and translocated populations: Implications for conservation.* Evolutionary Applications, 2019. **12**(3): p. 622-635.

22. Stumpf, R.M., et al., *Microbiomes, metagenomics, and primate conservation: New strategies, tools, and applications.* Biological Conservation, 2016. **199**: p. 56-66.

23. Weller, R. and D.M. Ward, *Selective Recovery of 16S rRNA Sequences from Natural Microbial Communities in the Form of cDNA.* Applied and Environmental Microbiology, 1989. **55**(7): p. 1818-1822.

24. Harwani, D., *The Great Plate Count Anomaly and the Unculturable Bacteria.* International Journal of Scientific Research, 2012. **2**: p. 350-351.

25. Carini, P., *A "Cultural" Renaissance: Genomics Breathes New Life into an Old Craft*. 2019.

26. Fenske, G.J., et al., *Integration of culture-dependent and independent methods provides a more coherent picture of the pig gut microbiome.* FEMS Microbiology Ecology, 2020. **96**(3).

27. SenGupta, D.J., et al., *Whole-Genome Sequencing for High-Resolution Investigation of Methicillin-Resistant Staphylococcus aureus Epidemiology and Genome Plasticity.* Journal of Clinical Microbiology, 2014. **52**(8): p. 2787-2796.

28. Carini, P., *A "Cultural" Renaissance: Genomics Breathes New Life into an Old Craft.* mSystems, 2019. **4**(3): p. e00092-19.

29. Weigelt, B., C.M. Ghajar, and M.J. Bissell, *The need for complex 3D culture models to unravel novel pathways and identify accurate biomarkers in breast cancer.* Advanced Drug Delivery Reviews, 2014. **69-70**: p. 42-51.

30. Gutleben, J., et al., *The multi-omics promise in context: from sequence to microbial isolate.* Critical Reviews in Microbiology, 2018. **44**(2): p. 212-229.

31. Stegen, J.C., et al., *Groundwater–surface water mixing shifts ecological assembly processes and stimulates organic carbon turnover.* Nature Communications, 2016. **7**(1): p. 11237.

32. Yan, L., et al., *Environmental selection shapes the formation of near-surface groundwater microbiomes.* Water Research, 2020. **170**: p. 115341.

33. Fredensborg, B.L., et al., *Parasites modulate the gut-microbiome in insects: A proof-of-concept study.* PLOS ONE, 2020. **15**(1): p. e0227561.

34. Cerqueira, G.M. and A.Y. Peleg, *Insights into Acinetobacter baumannii pathogenicity.* IUBMB Life, 2011. **63**(12): p. 1055-1060.

35. D'Argenio, V. and F. Salvatore, *The role of the gut microbiome in the healthy adult status.* Clinica Chimica Acta, 2015. **451**(Part A): p. 97-102.

36. Jin Song, S., et al., *Engineering the microbiome for animal health and conservation.* Experimental Biology And Medicine (Maywood, N.J.), 2019.

37. Cong, J. and X. Zhang, *How human microbiome talks to health and disease.* European Journal Of Clinical Microbiology & Infectious Diseases: Official Publication Of The European Society Of Clinical Microbiology, 2018. **37**(9): p. 1595-1601.

38. Gabriele, B., et al., *Microbiome definition re-visited: old concepts and new challenges.* Microbiome, 2020. **8**(1): p. 1-22.

39. Knight, R., et al., *Best practices for analysing microbiomes.* Nature Reviews Microbiology, 2018. **16**(7): p. 410-422.

40. Ilaria Laudadio, V.F., Francesca Palone, Laura Stronati, Salvatore Cucchiara, and Claudia Carissimi, *Quantitative Assessment of Shotgun Metagenomics and 16S rDNA Amplicon Sequencing in the Study of Human Gut Microbiome.* OMICS: A Journal of Integrative Biology, 2018. **22**(4): p. 248-254.

41. Vetrovsky, T. and P. Baldrian, *The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses.* PLoS One, 2013. **8**(2): p. e57923.

42. Mann, A.E., et al., *Comparison of the Bacterial Gut Microbiome of North American Triatoma spp. With and Without Trypanosoma cruzi.* Frontiers in Microbiology, 2020. **11**(364).

43. West, A.G.a.a.a.n., et al., *The microbiome in threatened species conservation.* Biological Conservation, 2019. **229**: p. 85-98.

44. Redford, K.H., et al., *Conservation and the Microbiome.* Conservation Biology, 2012. **26**(2): p. 195-197.

45. Allan, N., et al., *Conservation Implications of Shifting Gut Microbiomes in Captive-Reared Endangered Voles Intended for Reintroduction into the Wild*. 2018.

46. Wei, F., et al., *Conservation metagenomics: a new branch of conservation biology.* Science China Life Sciences, 2019. **62**(2): p. 168-178.

47. Cooke, M., G. Longley, and R. Gibson, *Spring association and microhabitat preferences of the Comal Springs riffle beetle (Heterelmis comalensis).* Southwestern Naturalist, 2015. **60**(1): p. 110-121.

48. USFWS, U.S.F.a.W.S., *Endangered and Threatened Wildlife and Plants; Designation of Critical Habitat for the Peck's Cave Amphipod, Comal Springs Dryopid Beetle, and Comal Springs Riffle Beetle*, in *71*. 2006: The Daily Journal of the United States Government. p. 40587-40621.

49. Huston, D. and J. Gibson, *Underwater Pupation by the Comal Springs Riffle Beetle, Heterelmis Comalensis Bosse, Tuff, and Brown, 1988 (Coleoptera: Elmidae), with an Update on Culture Techniques.* The Coleopterists Bulletin, 2015. **69**: p. 521-524.

50. Handelsman, J., *Metagenomics: application of genomics to uncultured microorganisms.* Microbiology and molecular biology reviews : MMBR, 2004. **68**(4): p. 669-685.

51. Hugenholtz, P., B.M. Goebel, and N.R. Pace, *Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity.* Journal of bacteriology, 1998. **180**(18): p. 4765-4774.

52. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nature Methods, 2016. **13**(7): p. 581-583.

53. Callahan, B., *Silva taxonomic training data formatted for DADA2 (Silva version 132).* Zenodo, 2018.

54. Dhariwal, A., et al., *MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data.* Nucleic Acids Research, 2017. **45**(W1): p. W180-W188.

55. Chong, J., et al., *Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data.* Nature Protocols, 2020. **15**(3): p. 799-821.

56. McManus, R., A. Ravenscraft, and W. Moore, *Bacterial Associates of a Gregarious Riparian Beetle With Explosive Defensive Chemistry.* Frontiers in microbiology, 2018. **9**: p. 2361-2361.

57. Segata, N., et al., *Metagenomic biomarker discovery and explanation.* Genome biology, 2011. **12**(6): p. R60-R60.

58. Kielak, A.M., et al., *The Ecology of Acidobacteria: Moving beyond Genes and Genomes.* Frontiers in Microbiology, 2016. **7**(744).

59. Li, L., et al., *Can closed artificial ecosystem have an impact on insect microbial community? A case study of yellow mealworm (Tenebrio molitor L.).* Ecological Engineering, 2016. **86**: p. 183-189.

60. Koch, H., et al., *Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus Nitrospira.* Proceedings of the National Academy of Sciences, 2015. **112**(36): p. 11371-11376.