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The Isolation and Characterization of the Microbial Flora in the Alimentary Canal of *Gromphadorhina portentosa* Based on rDNA Sequences.

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ABSTRACT

The Isolation and Characterization of the Microbial Flora in the Alimentary Canal of *Gromphadorhina portentosa* Based on rDNA Sequences

Amy Robertson

Multicellular organisms are not single individuals but carry a complex natural microflora with them. This complex's diversity and function can be considered a distinct ecosystem. Traditional methods of isolation and identification miss >90% of the actual diversity. This study uses the gut microflora of the Madagascar hissing roach, *Gromphadorhina portentosa*, as a model to examine this ecosystem. Isolated cultured bacteria were used to establish methods for identifying members of the microflora based on ribosomal RNA sequences. Universal primers for Eubacterial, Archaeal, and Eukaryotic 16s/18s rRNA were then used for PCR amplification of total DNA isolated from gut contents. Sequences from isolates were compared using BLAST, ClustalW, and other programs to recognize the isolates' identities and place them using a phylogenetic tree analysis. Eubacterial, Archaeal, and Eukaryotic organisms were found present in the hissing roach gut which can serve as a model ecosystem since it houses Eubacterial, Archaeal, and Eukaryotic organisms.

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CHAPTER 1

INTRODUCTION

Microbes are an often overlooked part of an ecosystem because of their small size and lack of morphological characters; however their presence is vital for every kind of known life. The number of microbial species that exists on the earth today is unknown, and classifying them becomes a massive project because of their small size, a traditional requirement for colony isolation, a requirement for a microbiological or biochemical biotype identification, and large diversity. Molecular techniques have yielded estimates that less than 10% of the organisms in an ecosystem have been identified using traditional methods (Keeton 2003). In the 19th century the development of pure culture techniques that allowed scientists to isolate and characterize prokaryotic organisms were developed by Robert Koch who stipulated that pure cultures of the pathogenic organism must be isolated from its host and grown in pure culture (Keeton 2003). With the development of molecular techniques such as PCR and rapid DNA sequencing, microbial diversity based on DNA sequences greatly simplified the identification of bacteria. Techniques comparing ribosomal DNA sequences were used to identify a third domain of life, Archaea, and establish a molecular-sequence based phylogenetic tree that could be used to relate all organisms (Pace 1997). This information was based on the fact that rDNA sequences are among the most conserved elements in living things. rDNAs from different organisms were sequenced using primers specific for each domain, and the alignment of the sequences was analyzed using a technique known as oligonucleotide cataloging. Oligonucleotide cataloging takes an RNA molecule and breaks it up into its basic components of (20) oligonucleotides by slicing at every guanine base using ribonuclease T_1 and then analyzing the subfragments.

Differences between the sequences were used to relate organisms (Woese 1987). With these data a molecular-based phylogenetic tree capable of classifying all organisms was created. The constructed tree demonstrated three primary lines of descent or domains named Eubacteria, Eukarya, and Archaea. The original rDNA sequences were then able to be rebuilt and were matched up based on their similarity. As a result, the relationships between all organisms were described as shown in Figure 1 (Morell 1997, Pace 1997).

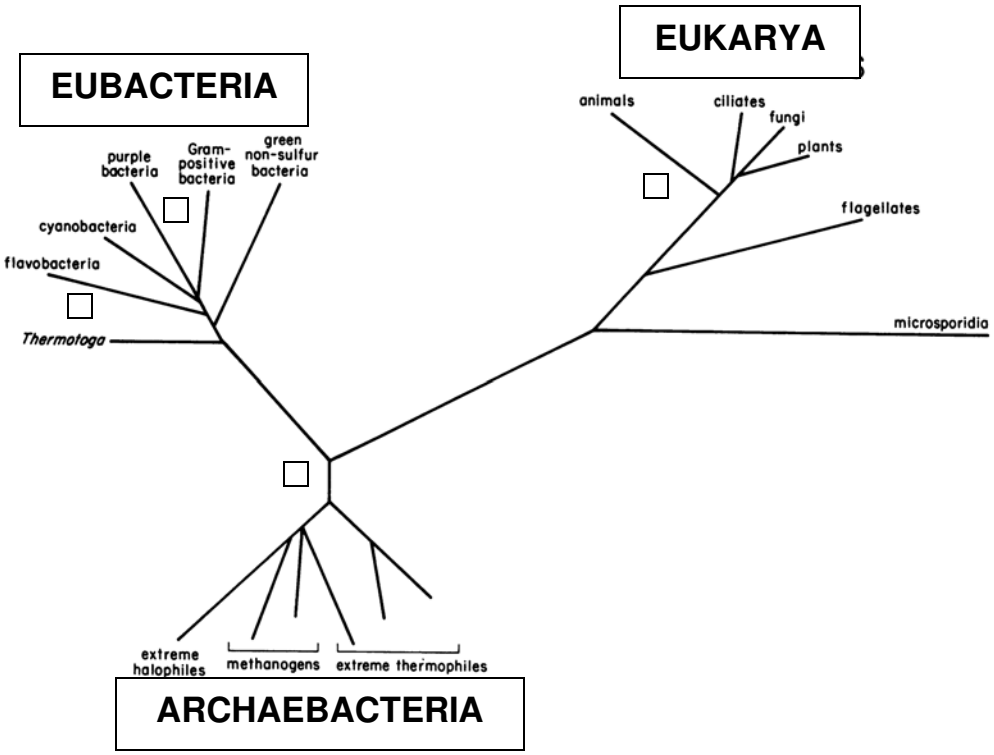


Figure 1 Universal Phylogenetic Tree Determined from rRNA Sequence Comparison (Adapted from Woese 1987). □ represents organisms that were obtained from PCR and cloning in my research in relation to this tree.

The compared ribosomal RNA sequences between bacterial domains (Archaea and Eubacteria) were also used to construct a phylogenetic tree that related known bacteria. The constructed tree suggested that Eubacteria are monophyletic as shown in Figure 2.

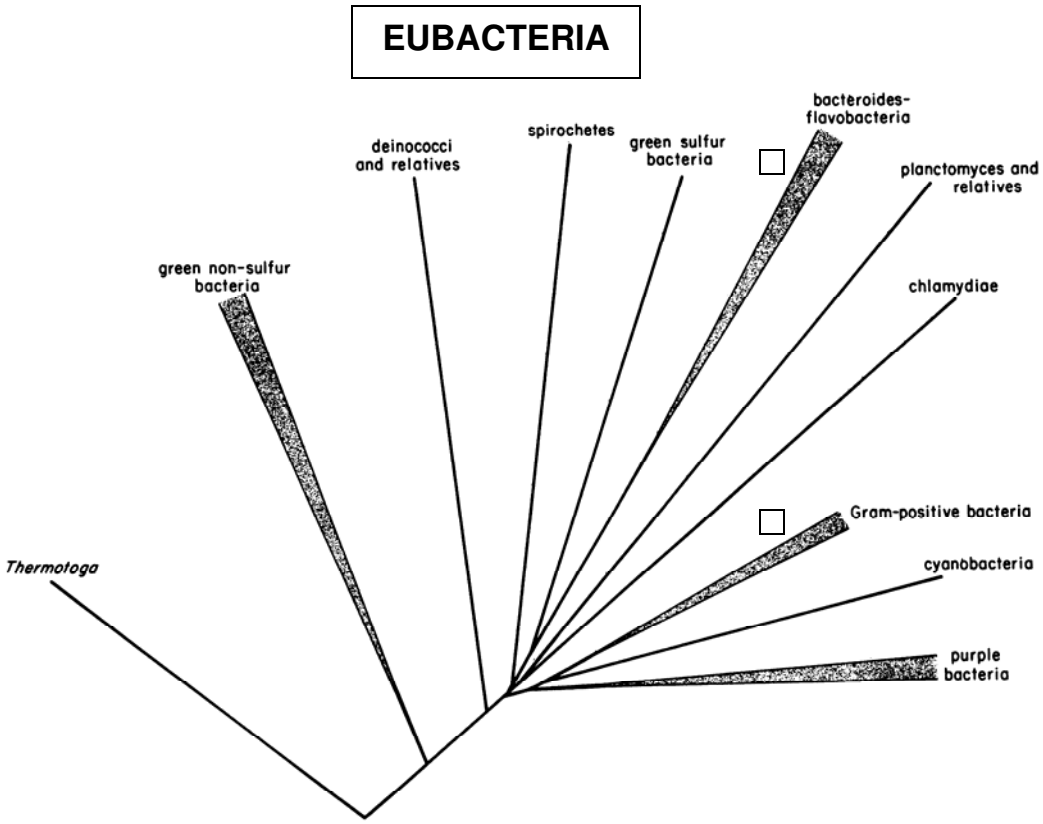


Figure 2 Eubacterial Phylogenetic Tree Based on 16s rRNA Sequence Comparison (Adapted from Woese 1987). □ represents organisms that were obtained from PCR and cloning in my research in relation to this tree.

With the advent of polymerase chain reaction (PCR) techniques, these sequences were used to investigate the diversity of organisms in environmental or ecological systems (Keeton 2003). However, in every ecosystem examined the diversity of microorganisms had been grossly underestimated using traditional techniques. PCR techniques indicated that greater than 90% of microorganisms were not being described in these systems (Pace 1997).

All organisms including vertebrates and invertebrates alike harbor a variety of bacteria, archaeabacteria, and eukaryotes in their digestive tracts. In some organisms, such as cows and termites, these microflora have been shown to provide necessary functions in the assimilation of food. Questions still remain concerning the diversity and function of these microflora in the gut.

For example, what is the diversity of microbes that inhabit it and what is their function to the general physiology of the host organism? Could this be considered a contained ecosystem that can be used to explore ecological questions about the interactions of literally billions of organisms in complex relations? Insects are good candidates as host organisms because they are cheap, easy to obtain and rear, and can be easily manipulated in a laboratory.

Roaches are candidate organisms because some species are large, easy to manipulate and are general foraging omnivores. The gut microflora of two species of cockroach have been examined (Cruden and Markovetz 1987). *Periplaneta americana* and *Eublaberus posticus* have a diverse diet that includes paper, bread, fruit, fish, putrid sake, cloth, hides, and hair. Over 100 different bacteria have been isolated from or passed through the gut of the cockroach. However, these microorganisms were isolated from feces or intestine and regarded as normal gut flora. Although the foregut consists of 50% of the total gut volume, not many microorganisms were found to colonize the foregut because its pH of 5.4 is unfavorable for microbial activities. Bacterial isolates from the foregut were not even identified (Cruden and Markovetz 1987).

The midgut, in contrast, showed quite a bit of microbial density. Approximately 10^8 colonies per midgut were counted when incubated aerobically and 3×10^8 colonies were isolated when incubated anaerobically. A dilution series isolated on medium containing carboxymethyl cellulose demonstrated that the midgut of both roach species contained 10^6 bacteria that grew under aerobic conditions and 10^7 that grew anaerobically. The most common microbes that were isolated from the midgut included anaerobes, *Enterobacter agglomerans* and *Klebsiella oxytoca,* and *Citrobacter freundii*. Electron microscopic examination of the midgut also showed that several types of insects harbor bacteria between the peritrophic membrane and midgut epithelium with occasional protozoa and spirochetes also present (Cruden and Markovetz 1987).

The hindgut in contrast, only harbors anaerobic bacteria. The bacteria consistently isolated included organisms *Clostridium sporogenes, Fusobacterium varium, Eubacterium moniliforme, Peptococcus variabilis, Peptostreptococcus productus,* and *Bacteriodes sp*. Bacteria frequently isolated from the cockroach hindgut include *Acidaminococcus fermentans, Propionibacterium avidum, Bifidobacterium sp., Clostridium bifermentans, Lactobacillus sp., Butyrivibrio sp., Coprococcus sp.,* and *Ruminococcus sp.* Even though these bacteria can be identified by species, many of the isolates cannot, especially when nonstandard enrichments are used such as stimulation with an inorganic phosphate. In addition to bacterial species in the hindgut, various types of ciliated protozoans were present as well as methanogens that live within the protozoans. The protozoans comprised approximately 0.2% of the population in the hindgut lumen. Methanogens included organisms with an ultrastructure similar to the genus *Methanospirillium* (Cruden and Markovetz 1987).

In addition to bacterial identification in the cockroach gut, ecological studies were done on the methanogens and ciliated protozoans. These studies investigated how methanogens that resemble organisms from the genus *Methanobacter* that live in the ciliated protozoan *Nyctotherus ovalis* in the gut of the American cockroach *Periplaneta americana* form a mutualistic relationship between the two organisms (Gijzen et al. 1991). The presence of the protozoan in the alimentary canal of the cockroach represents a production of methane. When metronidazole that inhibits methanogenesis was added to the cockroach drinking water significant changes were observed. Metronidazole was added for a period of 3 months and caused complete inhibition of methane production. Metronidazole was also added for 8 days and the *N. ovalis* cells were monitored every day and the protozoan was eliminated from the roach hindgut after 3 days. These studies show that the roach gut or alimentary canal can serve

as an isolated ecosystem and that the elimination of even one or two of the organisms can alter the system (Gijzen et al. 1991).

This system can also be used to investigate how the organisms interact with the host (ecosystem) (Gijzen et al. 1991). The elimination of *N. ovalis* from the cockroach hindgut demonstrated that when the cockroaches were raised without *N. ovalis*, they showed reduced body weight, increased generation time, and absence of methane production (Gijzen and Barugahare 1992). When the protozoan-free roaches were fed a hindgut suspension that contained *N. ovalis* as well as methanogens, methane production became normal and insect weight was reestablished during the second generation of insects. When protozoan-free roaches were fed a hindgut suspension with protozoans but no *N. ovalis* methane production grew to only 20% of the normal level. Roaches fed a suspension of bromoethanesulfonic acid reduced methane production to 2% of the normal level. This caused a shift in the hindgut fermentation pattern as well as an increase in propionate production. This shows that the ciliated protozoan *N. ovalis* plays a major role in methane production in the cockroach gut as well as in metabolism. This specific organism is of ecological importance because it contributes greatly to the overall metabolism and nutrition of the cockroach. Roaches are thought to be a major contributor of much of the world's methane. The interaction of these endosymbionts with each other and their host may point to new ways of how to control insects and demonstrate their importance to the ecosystem (Gijzen and Barugahare 1992).

Many of these studies were done in the 1980s and early 1990s before advanced molecular techniques were known. These early studies were based on Koch's principle of pure cultures/isolation and were limited because only a handful of microorganisms can be cultured using traditional methods (Pace 1997, Keeton 2003). Molecular techniques have allowed

biologists to greatly expand the classification and identification of organisms without these limitations (Pace 1997).

The early studies of environmentally isolated microbes were limited to pure cultures that can be grown in the laboratory; however, most of the isolates could not be identified and characterized through this system. Experiments on different environments have shown that more than 99% of microorganisms cannot be cultured by traditional techniques. With the development of rRNA sequencing to classify microbes, only the rRNA sequence is required to identify the prokaryote and be placed on the phylogenetic tree (Pace 1997). Ribosomal genes can be used because of their highly conserved function. Ribosomal sequences obtained by cloning DNA that has been isolated directly from the organism must be sorted from other isolates through a cloning procedure that allows each one to be sequenced. Ribosomal RNA's highly conserved nature allows for the use of "universal" PCR primers capable of annealing to conserved rRNA sequences from the three phylogenetic domains. The sequences can be compared with known sequences, analyzed, and placed on the phylogenetic tree based on the similarities in the sequences (Pace 1997).

A study that estimated bacterial biodiversity was done using rDNA PCR primers (Marchesi et.al. 1998). In the past, PCR primers often failed to work with some of the samples such as deep sea sediments, oral bacteria, and epilithon (biofilms that are associated with stones in moving water environments). Other primers were designed that were universal for Domain *Eubacteria* based on previous research on bacterial evolution. As a result of the primer redesign, a new array of bacterial species was identified. These included organisms from the genera *Micrococcus* and *Eubacterium* in addition to δ-proteobacteria and bacteria from moving water

samples and deep sea sediments. This study was the first that used universal Eubacterial primers for PCR amplification that amplified a larger number of bacterial genes (Marchesi et.al. 1998)

With the ability to identify increasing diverse prokaryotic species, attention was turned to eukaryotic species. The biodiversity of eukaryotic phytoplankton in the oceans revealed a vast array of eukaryotic organisms through the use of 16S small subunit rRNA amplification (Moriera and López-García 2002). It was previously shown within the Eubacteria domain that 13 divisions have been cataloged. There are, however, many organisms that have not been cultured within the two major Archaeal kingdoms Chrenarchaeota and Euryarchaeota. Several plastid genes were amplified which allowed the identification of a large number of photosynthetic lineages related to the classes Bacillaryophyceae, Cryptophyceae, Prymnesiophyceae, Chrysophyceae, and Prasinophyceae (Moreira and López-García 2002). This study also uncovered many new lineages as well. Some of these were affiliated to non-photosynthetic groups, one *Pseudo-nitzschia* like diatom, dinoflagellate phylotypes, and lineages that are not closely related to any known organisms. The most striking discovery was the assemblage of very diverse sequences that comprised the majority of their clones. These sequences formed two distinct clusters within the alveolates. This study demonstrates that the ocean is a vast ecosystem that contains microscopic eukaryotic organisms in greater diversity than previously thought (Moreira and López-García 2002).

Studies done to determine the biodiversity of microbes in the oceans have assessed marine microorganism diversity though sequencing ribosomal genes through the use of PCR amplification and have identified 20 major phyla in Bacteria and Archaea plus thousands of new taxonomic units. Larger microbes that were sequenced and divided into 5000 autotrophic and 1500 species based on morphology or outward characteristics (Falkowski and de Vargas 2004).

Whole genome sequencing is another technique that has been applied to environmental-pooled DNA samples (Venter et al. 2004). Picoplankton, or marine eukaryotes are thought to be the most abundant eukaryotes on earth, thus they would have a very wide range of genetic diversity. Total DNA sequencing and computational genomics has helped to identify more than 1.2 million new genes from organisms isolated from the Sargasso Sea. (Díez, Pedrós-Alió and Massana 2001, Venter 2004). Recently, approximately 1.6 million DNA sequences have been obtained from the Sargasso Sea and analyzed (Venter et al. 2001). The analysis first focused on the well-sequenced genomes by characterizing scaffolds, or proteins that remain when chromosomes are digested away (Lawrence 2001). The results were 333 scaffolds that comprised 2226 contigs (DNA sequences that are assembled from overlapping shorter sequences to form a continuous one) (Lackey 2001) that spanned 30.9 megabase (mbp) pairs. This accounted for roughly 410,000 reads, or 25% of the assembled data set. However, PCR studies have been discovered to be somewhat biased because not all genes will amplify with the same universal primers. The researchers, using the shotgun sequencing method, identified 1164 16s rRNA genes (Venter et al. 2004). Currently, there have been 36 to 38 phylogenetic divisions of microbes discovered based on 15,000 rRNA sequences from both cultured and environmental organisms. However, only 13 of these divisions have been encountered in environmental surveys and have not been able to be cultured using traditional methods. Some of these sequence-defined divisions have <10 sequences to represent them so the diversity of these organisms is vastly unknown (Dojka et al. 2000).

These studies still leave a lot of questions unanswered. What is the total diversity of bacteria, archaeabacteria, and eukaryotes in the environment? Could a more comprehensive description of species in the insect alimentary canal be identified through the use of rDNA

sequencing and PCR instead of traditional culturing methods? Could the insect gut serve as a model ecosystem?

My project is to describe the components of the alimentary canal of insects by examining the biodiversity of microbes present, identify them based on sequences from the extraction of rDNA, and develop this organismal complexity as an isolated ecosystem. The above studies provide evidence that it is possible to obtain many ribosomal genes from organisms comprising the three phylogenetic domains. The PCR product in this experiment should yield three different size products using specific primers for the Eukarytic, Eubacteria, and Archaeal domains.

I examined the types of microflora in the alimentary canal of the Madagascar hissing roach, *Gromphadorhina portentosa*, through the use of PCR, cloning, and sequencing. The gut microflora of insects has not been investigated significantly using these molecular techniques and this study will attempt to answer the question of what kinds of microflora can be found from the gut of an insect. Also, could an insect alimentary canal serve as a model for an ecosystem? This ecosystem would comprise organisms from all three phylogenetic domains and would explore the network of symbiotic relationships between organisms.

My working hypothesis states that when the biodiversity of microbes present in the alimentary canal of *Gromphadorhina portentosa* is examined, organisms comprising all three phylogenetic domains are present. These organisms can then be compared with other sequences from other organisms and placed on the phylogenetic tree produced by Woese that contains all three domains of life (Eubacteria, Archaeabacteria, and Eukarya). A prediction is that there will be some species of microflora that have not been identified, or if they have been identified, little is known about them. I identified bacteria that were isolated using traditional methods and then used total DNA from the roach's gut for PCR with universal primers, cloning, and DNA

sequencing. Sequences obtained were analyzed using BLAST, ClustalW, and other programs and placed on the phylogenetic tree comprising all three phylogenetic domains based on their similarities with other sequences.

CHAPTER 2

MATERIALS AND METHODS

Biochemicals and Bioinformatics Tools

For roach gut tissue extraction, insect saline (Appendix B) was used to clean roach tissue and the gut was extracted with dissecting scissors. DNA was isolated from the gut tissue using DNAzol (Molecular Research Center Inc.,Cincinnati, OH) chloroform (Fisher Scientific, Fairlawn, NJ), phenol (Sigma Aldrich, St. Louis, MO), lysozyme (100 µg/µl) (Sigma-Aldrich, St. Louis, MO), 10% SDS, and Proteinase K (100 µg/µl). Isolated DNA was stored in TE buffer (recipe in Appendix B).

ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich, St.Louis, MO) and DIUF water, primers designed from Operon Biotechnologies Inc. (Huntsville, AL) and Sigma Genosys Custom Oligos (Sigma-Aldrich Co., The Woodlands, TX), and extracted template DNA were used for PCR. PCR products were extracted from the 1% gel using GenElute Agarose Spin Columns (Sigma-Aldrich, St. Louis, MO) and purified using 7.5 M NH_4oAc and 95% EtOH. Blue dye (Finnzymes, Espoo, Finland) was used to determine the DNA's location on the gel. Molecular weight markers (DNA ladders) included λ/*HindIII*, ExACTGene Low Range Plus DNA Ladder (Bioline USA Inc., Randolph, MA), and λDNA-*HindIII*/øx-*HaeIII* (Finnzymes, Espoo, Finland)).

Cloning used 38i and 28i (5 µg/ml) LITMUS cloning vectors (New England Biolabs, Beverly, MA), purified PCR product, *BamH* 1 enzyme (2500 units) (Fisher Scientific, Pittsburg, PA), and *Sal* 1 enzyme (2000 units) (Fisher Scientific, Pittsburg, PA). QIAGEN® PCR Cloning

Kit (40 rxns) (Qiagen Inc., Valencia, CA) was used and included pDrive Cloning Vector (2.0 µg, 50 ng/µl).

Ligation reactions used Quick Stick Ligation Kit (50 reactions) (Bioline USA Inc., Randolph, MA) containing T4 DNA ligase (50 µl). Another kit (Promega Corporation, Madison, WI) contained pGEM®-T Easy Vector (1.2 µg).

Transformation reactions were done using One Shot® Mach1™-T1R Chemically Competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA), SOC medium (Invitrogen Life Technologies, Carlsbad, CA), and X-gal (20 µg/µl) (Fisher Biotech, Fair Lawn, NJ). Plasmid DNA isolations used QIAprep® Spin Miniprep Kits (50 rxns) (Qiagen Inc., Valencia, CA) containing spin columns (50),

LB broth (Fisher Scientific Inc., Fair Lawn, NJ) was used with ampicillin (25 µg/µl), LB agar (Fisher Scientific Inc., Fair Lawn, NJ). Ampicillin (Fisher Scientific, Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO)) and X-gal plates were prepared according to the recipes in Appendix B.

Gels were run with 1X TBE buffer (prepared according to recipe in Appendix B) and 1% agarose (Fisher Biotech Inc., Fairlawn, NJ). These gels were stained in 10 µg/µl EtBr (Fisher Scientific, Fair Lawn, NJ), (EtBr was also prepared according to the recipe in Appendix B). The gels were photographed using the program LabWorks 4.0. PCR products were further purified for sequencing using GENECLEAN® *Turbo* Kit (100 preps) (Qbiogene Inc., Solon, OH).

For DNA sequencing, NCBI's BLAST website was used to identify organisms and construct phylogenetic trees, ClustalW (EMBL-EBI) was used to align the sequences, and the University of Tennessee, Knoxville's services were used to sequence the DNA.

Maintenance of *Gromphadorhina portentosa*

Madagascar hissing roaches, *Gromphadorhina portentosa*, are large wingless insects native to the island of Madagascar off the eastern coast of Africa. They live on the forest floor as omnivorous scavengers. One of the distinguishing characteristics of this insect is its ability to produce sound through spiracles in its abdomen. This occurs when the roach is disturbed by a predator or when males come in contact with each other (Clark and Shanklin 1995).

G. portentosa colonies originated from Ohio State University's entomology department and were maintained since 1994 at East Tennessee State University. Roaches were fed dry dog food and tap water *ad libitum* in a plastic container. Colonies were kept at 27° C in constant darkness. *G. portentosa* is shown in Figure 3.

Figure 3 Laboratory-Raised *G. portentosa*

Methods for Microflora Identification

Bacterial Isolation from Roach Tissue and Feces

Microorganism isolation was from feces and gut tissue collected from immature roaches. Once extracted, the feces were placed into a test tube with 1 ml BHI broth and mixed to remove organisms. The broth was streaked onto four plates—two blood agar and two nutrient agar. One blood agar and one nutrient agar plate were placed into an anaerobic chamber with a gas pack. Ten ml of water was added to this gas pack (containing $NaBH_4$) causing O_2 to be removed. The other two plates were placed into a jar with a lit candle that resulted in elevated CO_2 levels.

The gut and contents were similarly extracted and placed into a sterile tissue homogenizer with 2 ml BHI broth and ground. The extracted samples were streaked onto blood agar and nutrient agar plates and placed in an aerobic jar with a candle to raise CO_2 levels. Both the candle jar and the anaerobic chamber were incubated at 30° C. The culture conditions for the samples are shown in Table 1.

Table 1 Reaction Conditions for Tissue and Feces

	Blood Agar	Nutrient Agar
Anaerobic chamber	Feces	Feces
Anaerobic chamber	Roach #1 Tissue	Roach #1 Tissue
Candle Jar	Feces	Feces
Candle Jar	Roach #2 Tissue	Roach #2 Tissue

The ground up tissue and feces samples were individually mixed with BHI (800 µl of each sample) and placed into glass vials with 200 µl 75% glycerol (to prevent freezing at very low temperatures) (18.75% glycerol final concentration). Samples were stored at -80° C.

Single Colony PCR

Single colony PCR was performed on 10 of the individual colonies that grew (10 different ones). For this reaction, ten 1.5 ml microcentrifuge tubes were labeled 1-10 and 10 µl PCR water was added to each of them. In each of these tubes a single different bacterial colony was added using an inoculation needle and was mixed thoroughly in the water. The plate conditions for these tube samples are displayed in Table 2.

Table 2 Plate Conditions for Tube Samples

Tube #1	Feces/Anaerobic Chamber/Blood Agar
Tube #2	Feces/Anaerobic Chamber/Blood Agar
Tube #3	Feces/Anaerobic Chamber/Blood Agar
Tube #4	Roach #2 Tissue/Candle Jar with Elevated CO_2/Nutrient Agar
Tube #5	Roach #2 Tissue/Candle Jar with Elevated CO_2/Blood Agar
Tube #6	Feces/Anaerobic Chamber/Blood Agar
Tube #7	Roach #2 Tissue/Candle Jar with Elevated CO_2/Blood Agar
Tube #8	Roach #1 Tissue/Anaerobic Chamber/Blood Agar
Tube #9	Roach #1 Tissue/Anaerobic Chamber/Blood Agar
Tube #10	Roach #1 Tissue/Anaerobic Chamber/Blood Agar

For the PCR reaction, 1 µl of liquid from each sample was placed into a clean 0.2 ml PCR tube and mixed with 1 µl each of FB and RS Eubacterial primers (*BamH1* and *Sal1* restriction sites), 12.5 ReadyMix™ Taq PCR Reaction mix, and 9.5 µl PCR water. These reactions were mixed by vortexing and placed into a thermocycler. The PCR cycles are displayed in Table 3.

Table 3 Cycles for Single Clony PCR

95°C/2 min			1 cycle
95°C/1 min	50°C/2 min	72°C/2 min	30 cycles
4°C/park			

When the PCR cycles were complete, 10 µl of each PCR product sample was run in two 1% agarose gels at 104 volts for 45 minutes. Gels were stained in 200 ml 1X TBE buffer with 10 µl EtBr (10 µg/µl) for 30 minutes. They were viewed on a transilluminator and photographed using LabWorks 4.0. The PCR products (bands) were cut out from the gel and purified by adding DIUF water (90 µl) to each sample and mixing with 250 µl ice cold 95% EtOH to precipitate the DNA. The samples were placed at -20° C overnight and spun for 15 minutes at 14,000 rpm. The pellet was allowed to air dry briefly, and the DNA redissolved in 15 µl DIUF water. A repeat PCR reaction was performed using 1 µl DNA from the single colony dissolve (1 bacterial colony + 10 µl DIUF water), 12.5 µl ReadyMix™ Taq PCR Reaction mix, 2 µl FB and RS primers (Eubacterial with *BamH* 1 and *Sal* 1 sites), and 9.5 µl DIUF water. They were run in the thermocycler with the same cycles as Table 3, run on a gel, and purified using the PCR purification protocol.

Purification of PCR Products

Further purification of these products was performed using the GENECLEAN® *Turbo* Kit according to the kit instructions. For these reactions, 5 volumes of *Turbo* Salt Solution (75 µl) was added to each of 10 tubes. Each of these reactions was transferred to a separate *Turbo* cartridge in a 2 ml catch tube. The tubes were centrifuged for 5 seconds at 13,200 rpm. After centrifugation 500 µl prepared *TurboWash* was added to each tube and centrifuged for 13,000 rpm for 5 seconds. The flowthrough was discarded and the tubes centrifuged for an additional 4 minutes (13,200 rpm) to force the remaining wash through the tube. After this remaining wash was discarded, the filters were placed into new, clean *Turbo* catch tubes and 50 µl Turbo Elution Solution was added to the center of each tube and allowed to sit for 5 minutes at room

temperature. The tubes were centrifuged for 1 minute at 13,200 rpm and eluted was DNA stored in the freezer at -20° C. The DNA was further purified for sequencing using the 95% EtOH precipitation method used previously. These PCR products were run in 5µl aliquots on a 1% agarose gel to demonstrate that DNA was present. Samples with DNA present were sent for sequencing (UT Knoxville sequencing lab). Eubacterial and Eukaryotic primers (Table 10) (with *BamH* 1 and *Sal* 1 sites) were diluted to 5 µM concentration according to Table 4 in order to be used for sequencing from single colony PCR.

Table 4 Dilution of Eubacterial and Eukaryotic Primers

Primer	Amount Stock Added	Amount DIUF H_20 Added	Total Volume
Eub 27FB	5 µl	28.6 µl	33.9 µl
Eub 1492RS	5 µl	32.9 µl	37.9 µl
Euk FB	5 µl	36.2 µl	41.2 µl
Euk RS	5 µl	31.9 µl	36.9 µl

Isolation of Chromosomal DNA from Bacterial Colonies

Two plates were obtained, one plate from the anaerobic jar and one from the candle jar the bacterial colonies scraped off of them, and the bacteria washed in 500 µl mM Tris (pH 8.0). After washing, the liquid was poured off and the bacteria were stored at -20° C. The colonies were resuspended in two tubes each with 160 µl 25% sucrose solution in 50 mM Tris in 1.5 ml microcentrifuge tubes before they were finished thawing. Lysozyme (60 µl, 10 mg/ml) was added to each tube, shaken to mix, and placed in a hot water bath at 37° C for 30 minutes. After incubation, 67 µl 0.25 M EDTA was added to each tube as well as 50 µl 10% SDS and 50 µl Proteinase K (1 mg/ml) to break up cell membranes . The tubes were inverted gently until the solution became viscous and clear. They were incubated in the hot water bath at 37° C for 30 minutes. After incubation, 150 µl 50 mM Tris (pH 8.0) to stabilize pH was added and the tubes

mixed gently. Phenol (500 µl) was then added remove and denature proteins from the DNA, the tubes mixed vigorously, and placed on ice 1-2 minutes. They were centrifuged 5 minutes at 15,000 rpm at 4° C. The aqueous (top) layer from each tube was saved and re-extracted with phenol (450 µl). The tubes were mixed vigorously, incubated on ice 1-2 minutes, spun at 4° C at 15,000 rpm for 5 minutes, and the aqueous layer saved. Chloroform (450 µl) was added to each of the tubes to remove the phenol. They were shaken vigorously, incubated on ice 1-2 minutes, and spun at 4° C at 15,000 rpm for 2.5 minutes. The aqueous layer was saved and re-extracted. Chloroform was treated as before. The aqueous layer from each tube was saved and 1/10 volume 10 M NH_4oAc was added to convert the DNA to a salt instead of an acid. Ice cold 100% EtOH was added to precipitate the DNA salt. The tubes were mixed well and the DNA was spooled out with a 0.1-10 µl plastic pipet tip and placed into 1.5 ml tubes with 400 µl 70% EtOH. These tubes were spun at 4° C at 15,000 rpm for 30 seconds. The EtOH was aspirated off using a Pasteur pipet and the DNA briefly allowed to air dry. It was resuspended in 100 µl 0.1X TE buffer (pH 8.0) to dissolve the DNA. The tubes were stored at 4° C overnight. The chromosomal DNA was diluted 1/5000. Leftover unspooled DNA from the tube was spun in the centrifuge for 15 minutes at 10,000 rpm, the EtOH aspirated off using a Pasteur pipet, briefly allowed to air dry, and resuspended in 50 µl DIUF water.

PCR Reactions Using Chromosomal DNA

PCR reactions were performed using the diluted spooled chromosomal DNA and the leftover unspoiled DNA from the extraction. For this reaction, primers (with *BamH* 1 and *Sal* 1 sites, Table 10) representing the three phylogenetic domains (Eubacteria, Archaea, Eukarya) were used. Six 0.5 ml microcentrifuge tubes were obtained and labeled accordingly. These reactions were mixed up and run with the same PCR cycles as Table 5 demonstrates.

Table 5 PCR Reactions for Spooled and Unspooled Chromosomal DNA

	Amount DNA	Amount Taq Mix	Amount Primers	Amount Water Used	Total Volume
Eub Spooled Chromosomal DNA	5 µl	12.5 µl	1 µl FB 1 µl RS	5.5 µl	25 µl
Eub Unspooled Chromsomal DNA	3 µl	12.5 µl	1 µl FB 1 µl RS	7.5 µl	25 µl
Arch Spooled Chromosomal DNA	5 µl	12.5 µl	1 µl FB 1 µl RS	5.5 µl	25 µl
Arch Unspooled Chromsomal DNA	5 µl	12.5 µl	1 µl FB 1 µl RS	5.5 µl	25 µl
Euk Spooled Chromosomal DNA	5 µl	12.5 µl	1 µl FB 1 µl RS	5.5 µl	25 µl
Euk Unspooled Chromsomal DNA	5 µl	12.5 µl	1 µl FB 1 µl RS	5.5 µl	25 µl

After the PCR cycles were complete, the six samples were run on a 1% agarose gel at 104 volts for 45 minutes. The gel was stained in 200 ml TBE + 20 µl EtBr (10 µg/µl) for 30 minutes. The PCR products were purified using the same process as above.

Ligation, Electroporation, and Transformation Reactions

For ligation, five different reactions were performed with the samples shown in Table 6. The reactions were vortexed and placed in the refrigerator at 4º C overnight.

Table 6 Ligation Reaction Set Up

	2x Rapid Ligation Buffer	pGEM® -T Vector	PCR DNA from Tubes	T4 DNA Ligase	dH_2O Added
Eub Sp. Chr.	5 µl	1 µl	3 µl	1 µl	--
Euk Sp. Chr.	5 µl	1 µl	3 µl	1 µl	--
Eub Unsp. Chr.	5 µl	1 µl	3 µl	1 µl	--
+ Control	5 µl	1 µl	--	1 µl	1 µl
- Control	5 µl	1 µl	--	1 µl	3 µl

Four vials (100 µl each) of DH5α electrocompetent *E. coli* cells were thawed on ice. One of the vials was divided into 50 µl cells each for the controls. After thawing, 1.5 µl of ligation reaction was added to each vial and each reaction was transferred to 2 mm electroporation cuvets. Electroporation was performed at 2500 volts. The time constant for each of the different reactions was recorded as shown in Table 7.

Table 7 Time Constants for Ligation Reactions

Reaction	Eub Sp. Chr.	Euk Sp. Chr.	Eub Unsp. Chr.	+ Control	- Control
Time Constant	4.8 s	4.4 s	4.8 s	5.2 s	5.2 s

Five 16 ml test tubes were obtained and 500 µl SOC medium was added to the cuvets, mixed, and then extracted using a Pasteur pipette. The mixture from each cuvet was then placed into the test tubes and allowed to incubate at 37º C for 1 hour. Eleven LB + amp plates were prepared using 75 µl IPTG and 25 µl X-gal on 9 of them. Two plates containing carbinicillin were also used. The IPTG was smeared on the plates and allowed to dry, and then the X-gal was added. After an hour the transformation reactions were diluted and inoculated onto the plates according to Table 8. The transformations were then placed in the incubator at 37° C overnight.

Table 8 Dilutions for Transformation Plates

	Vol/Plate	Type Plate	X-gal + IPTG	# 10^0 (Undiluted)	# 10^2
Eub Sp. Chr.	100/50 µl	LB + Amp ^{50}	Yes	1	2
Euk Sp. Chr.	50 µl	LB + Amp ^{50}	Yes	1	2
Eub Unsp. Chr.	50 µl	LB + Amp ^{50}	Yes	1	2
+ Control	50 µl	LB + Amp ^{50}	No	1	0
- Control	50 µl	LB + Amp ^{50}	No	1	0

Growth of Colonies and Plasmid Extraction

Three of the plates (Eub Sp. Chr., Euk Sp. Chr., and Eub Unsp. Chr.) were placed in the refrigerator overnight. Two and a half ml LB + 2.5 µl ampicillin (25 µg/µl) was added to each of thirty-two 18-ml test tubes. Each of 32 individual colonies was taken from these plates using an inoculation needle dipped in LB broth to ensure the bacteria would come off the plates more easily. Nine colonies came from the Eubacterial Unsp. Chr. plate, 4 colonies came from the Eukaryotic Sp. Chr., and 17 colonies came from the Eubacterial Sp. Chr. plate. The other two colonies were blue ones (controls that did not contain the 16s/18s ribosomal gene insert) that came from the Eubacterial Unsp. Chr. plate (10^0 dilution) and Eukaryotic Sp. Chr. plate (10^0 dilution). These tubes were placed in the shaking incubator at 200 rpm at 37° C overnight. Plasmids were isolated from the colonies that grew with QIAGEN QIAPrep® spin miniprep kits. At the end of the protocol, instead of allowing the samples to stand for 1 minute before centrifugation (elution step), they were allowed to stand 15 minutes.

Insert Confirmation Using Double Digests and PCR

A double digest reaction using *BamH* 1 and *Sal* 1 restriction enzymes was performed on the Eubacterial (Unsp. Chr.) and Eukaryotic (Sp. Chr.) samples. For these reactions, 7 µl DNA,

1 µl *BamH* 1 enzyme, 1 µl *Sal* 1 enzyme, 4 µl Buffer E, and 9 µl PCR + DIUF H_2O were mixed by pipetting in labeled 0.5 ml microcentrifuge tubes. The tubes were placed at 37° C overnight. The samples were run on two 1% agarose gels as above. PCR reactions were also performed with these 9 samples as well as the controls. The Eukaryotic samples were placed in the thermocycler and the cycles from Table 5 were used. For the Eubacterial samples, the cycles shown in Table 3 were used. These samples were run on 1% agarose gels as above. They were stained in 300 ml TBE with 20 µl EtBr (10 µg/µl) for 20 minutes. These samples were also purified using 2.5 volume ice cold 95% EtOH and 0.5 and 0.1 volumes NH_4oAc. Once these two reagents were added to the samples, they were stored in the freezer at -20° C. They were then centrifuged at 10,000 rpm for 15 minutes. The EtOH was aspirated off using a Pasteur pipet and 50 µl DIUF H_2O was added.

Purification of Samples and Backup PCR

The three PCR samples were purified as done previously using GENECLEAN® Turbo Kit, EtOH, and NH_4oAc. A repeat PCR reaction was also performed using the leftover unspooled chromosomal DNA from the extraction as well as a 1/5000 dilution of the spooled chromosomal DNA. A new PCR reaction was performed and a gel run with these products. The Eubacterial band was cut out and gel purified. Another PCR reaction was then run and the Eukaryotic band extracted (gel purified).

Analysis of Sequences

Sequences were copied from the BioEdit program (Ibis Therapeutics) into a Microsoft Word document. The FB sequences were taken for all 13 samples (Eub and Euk) and the first

500-900 base pairs were recopied. The unnecessary N's from the sequences were also deleted. The sequences were then placed into the BLAST website (NCBI), their relationships were observed, and phylogenetic trees were constructed from the relationships with other organisms. The edited sequences from these organisms were compared with the sample sequences in ClustalW (EMBL-EBI).

Extraction of DNA from Roach Gut Content

For DNA isolation, a large adult male *G. portentosa* was obtained and anesthetized in the freezer. The gut content was dissected in insect saline and weighed in a small, plastic tube. Four ml DNAzol was added and then ground with mortar and pestle. Four hundred µl 10% SDS were then added and ground more. This mixture was incubated at 65° C for 30 minutes. After incubation, 1.35 ml chloroform was added and centrifuged 20 minutes at 10,000 rpm. Approximately 4 ml of a brown liquid was collected and 4 ml chloroform and 4 ml phenol were added. The mixture was shaken and centrifuged for 20 minutes at 10,000 rpm. After centrifugation the mixture was placed in the refrigerator at 4º C. The mixture was measured and equal volumes of phenol and chloroform were added. The mixture was shaken and centrifuged for 20 minutes at 10,000 rpm. The brown supernatant was taken off and measured and equal volumes of phenol and chloroform were added again and centrifuged for 20 minutes at 10,000 rpm. The brown supernatant was collected and an equal volume of chilled isopropanol was added and stored in the refrigerator overnight. The mixture was centrifuged 20 minutes at 10,000 rpm and the pellet was redissolved in 1 ml TE buffer. The solution was aliquoted into two microfuge tubes. The DNA was precipitated by the addition of 60 µl NH_4oAc (7.5 M) and 1200 µl 95% EtOH. The tubes were refrigerated and then centrifuged for 20 minutes at 10,000

rpm in the tabletop centrifuge. The supernatant was discarded and 100 µl TE buffer was added to each tube to dissolve the DNA. The DNA solution was again extracted with 50 µl chloroform and 50 µl phenol and centrifuged 20 minutes at 10,000 rpm. The aqueous layer was reprecipitated with one-tenth volume of 7.5 M NH_4oAc and 2 volumes of 95% ethanol. After refrigeration, the DNA was pelleted by centrifugation 20 minutes at 10,000 rpm and the supernatant poured off. The pellet was dissolved in 200 µl TE buffer and further purified by the addition of 250 µl DNAzol and 75 µl chloroform. After centrifugation for 20 minutes at 14,000 rpm, the top layer (aqueous) was recovered. An equal volume of isopropanol (~500 µl) was added and the mixture was placed in the refrigerator overnight. It was centrifuged for 20 minutes at room temperature at 14,000 rpm and the pellet was resuspended in 50 µl TE buffer. Aliquots of 5 µl, 10 µl, and 20 µl were run on a 1% agarose gel.

Extraction of *E. coli* DNA as a Control

Escherichia coli DNA was isolated as a control for use with Eubacterial primers. A solution (10 ml) of LB broth was incubated overnight at 37°C with *E. coli* bacteria. The solution was spun to pellet the bacterial cells. After the cells were pelleted a 100 µl lysozyme solution was added and placed in a shaker at room temperature for 15 minutes. A volume of 800 µl of 10% SDS was added and the mixture incubated at 65°C for 20 minutes. A volume of 2 ml DNAzol was added followed by 750 µl chloroform. The mixture was centrifuged at 10,000 rpm for 20 minutes and the supernatant recovered (3 ml). An equal volume of isopropanol was added, placed in the refrigerator for 1 hour, and centrifuged for 20 minutes at 10,000 rpm. The supernatant was poured off and 100 µl TE buffer was added to dissolve the DNA. A 1% agarose gel was run with an aliquot of 10 µl *E. coli* DNA, 2 µl EtBr (10 µg/µl), and 2 µl blue dye.

Demonstration of DNA Presence

The presence of DNA was demonstrated by running samples on a 1% agarose gel (0.30 gram agarose and 30 ml 1X TBE buffer). The agarose was melted in buffer by boiling for 2 minutes and poured into a gel mold. After the gel had hardened, the DNA samples were loaded into the gel along with a molecular marker (λ cut with *Hind III*) with 2 µl EtBr (10 µg/µl) and 2 µl blue dye. After the dye was ¾ of the way down the gel it was stained in 200 ml TBE with 10 µl EtBr (10 µg/µl) for 15 minutes in the shaker incubator. It was viewed under the transilluminator and photographed using LabWorks 4.0.

DNA was quantified using a spectrophotometer and 0.5 ml glass cuvets. The first cuvet had 500 µl dH_2O and served as a blank. The second cuvet contained 5 µl DNA and 495 µl dH_2O. This DNA could then be used for PCR analysis.

PCR Amplification

PCR amplification was performed using several sets of primers representing the three phylogenetic domains (Eubacteria, Archaea, Eukarya). Initial sets of primers were used (Joplin 1998) as well as second and third sets (DeLong 1992). The initial sets of primers from Table 9 and the first ordered sets from Table 10 contained *BamH* 1 and *Sal* 1 restriction sites. The second ordered sets, however, contained only *BamH* 1 sites (Table 12). Primers were diluted accordingly (Tables 11 and 13) to obtain a 10 mM concentration.

Table 9 Initial Sets of Primers (Joplin, 1998)

Specific Domain	Primer Name	Primer Sequence (5' to 3')
Eubacteria 1a	63FB	GCGGGATTCCAGGCCTAACACATGCAAGTC
Eubacteria 1b	1357RS	GGCCGTCGACGGTTACCTTGTTACGACTT
Archaebacteria 1a	21FB	GCGGGATTCTTCTTCCGGTTGATCCYGCCGGA
Archaebacteria 1b	958RS	GGCCGTCGACYCCGGCGTTGAMTCCAATT
Eukarya 1a	Euk FB	GCGGGATTCAACCTGGTTGATCCTGCCAGT
Eukarya 1b	Euk RS	GGCCGTCGACTGATCCTTCTGGTTCACCTAC

Table 10 First Sets of Primers Ordered (Delong 1992 PNAS 89:5685-5689). Nucleotides in red represent *BamH* 1 sites and nucleotides in green represent *Sal* 1 sites.

Specific Domain	Primer Name	Primer Sequence (5' to 3')
Eubacteria 2a	27FB	GCGGGATCCAGAGTTTGATCCTGGCTCAG
Eubacteria 2b	1492RS	GGCCGTCGACGGTTACCTTGTTACGACTT
Archaebacteria 2a	21FB	GCGGGATCCTTCCGGTTGATCCYGCCGGA
Archaebacteria 2b	958RS	GGCCGTCGACYCCGGCGTTGAMTCCAATT
Eukarya 2a	Euk FB	GCGGGATCCAACCTGGTTGATCCTGCCAGT
Eukarya 2b	Euk RS	GGCCGTCGACTGATCCTTCTGCAGGTTCACCTAC

Table 11 Concentrations and Dilutions of First Sets of Stock Primers. When primers were sent 1 ml of PCR H_2O was added and then the primers were diluted to a 10 mM concentration.

Primer Name	Concentration	Amount of Stock Added	Amount of PCR H_2O Added to Make 10 mM Concentration
Eub 27FB 2a	33.6 nmol	10 µl	23.6 µl
Eub 1492RS 2b	37.9 nmol	10 µl	27.9 µl
Arch 21FB 2a	53.0 nmol	10 µl	43.0 µl
Arch 958RS 2b	59.7 nmol	10 µl	49.7 µl
Euk FB 2a	41.2 nmol	10 µl	31.2 µl
Euk RS 2b	36.9 nmol	10 µl	26.9 µl

Table 12 Second Sets of Primers Ordered (Delong 1992 PNAS 89:5685-5689). Nucleotides in red represent *BamH1* sites.

Specific Domain	Primer Name	Primer Sequence
Eubacteria 3a	27FB	GCG**GGATCC**AGAGTTTGATCCTGGCTCAG
Eubacteria 3b	1492RS	GCG**GGATCC**GGTTACCTTGTTACGACTT
Archaebacteria 3a	21FB	GCG**GGATCC**TTCCGGTTGATCCYGCCGGA
Archaebacteria 3b	958RS	GCG**GGATCC**YCCGGCGTTGAMTCCAATT
Eukarya 3a	Euk FB	GCG**GGATCC**AACCTGGTTGATCCTGCCAGT
Eukarya 3b	Euk RS	GCG**GGATCC**TGATCCTTCTGCAGGTTCACCTAC

Table 13 Concentrations and Dilutions of Second Sets of Stock Primers. When primers were sent 1 ml of PCR H_2O was added and then the primers were diluted.

Primer Name	Concentration	Amount of Stock Added	Amount of PCR H_2O Added to Make 10 mM Concentration
Eub 27FB 3a	70.65 nmol	10 µl	60.7 µl
Eub 1492RS 3b	67.81 nmol	10 µl	57.8 µl
Arch 21FB 3a	59.94 nmol	10 µl	49.9 µl
Arch 958RS 3b	79.17 nmol	10 µl	69.2 µl
Euk FB 3a	71.61 nmol	10 µl	61.6 µl
Euk RS 3b	82.16 nmol	10 µl	72.2 µl

PCR was performed using 12.5 µl ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma Aldrich), 5.5 µl PCR water, 5 µl DNA, and 1 µl each of FB and RS (forward and reverse) primers from each of the three phylogenetic domains (Eubacteria, Archaea, and Eukarya). These reactions were mixed and placed in the thermocycler according to the cycles first in Table 14 and then in Table 15. The samples were then placed into a 1% agarose gel with ExACTGene DNA ladder as a molecular weight standard and run for 45 minutes at 104 volts. The gels were stained in 200-300 ml 1X TBE with 10 µl EtBr for 20 minutes. They were viewed under the transilluminator and photographed using the program LabWorks 4.0. Fifty-seven PCR reactions were performed on DNA from Extractions #2-5.

Table 14 Initial Sets of PCR Cycles (Joplin 1998)

94°C/5 min			
94°C/1 min	50°C/2 min	65°C/2 min	5 cycles
94°C/1 min	55°C/1 min	72°C/1 min	35 cycles
72°C/5 min	4°C/park		

Table 15 Second Sets of PCR Cycles (Ueda et. al. 1995)

94° C/5 min			
94° C/30 sec	35° C/1 min	65° C/2 min	5 Cycles
94° C/30 sec	50° C/30 sec	72° C/1 min	30 Cycles
72° C/5 min	4° C/Park		

A gradient was also run to determine which annealing temperature was ideal for each of the primers (Eubacterial, Archaeal, Eukaryotic). Annealing temperatures for each of the three phylogenetic domains are displayed below in Table 16. The gradient set up was set up according to Table 17.

Table 16 Primer Annealing Temperatures

	FB	RS
Eubacteria	79.7° C	85.4° C
Archaebacteria	84.3° C	82.8° C
Eukarya	81.0° C	78.6° C

Table 17 Annealing Temperature Gradient Set Up for PCR. Gradient ranged from 78-86°C.

Eubacteria	Well 5	Well 6	Well 10	Well 11
	79.3° C	80.5° C	85.1° C	83.8° C
Archaeabacteria	Well 7	Well 8	Well 9	Well 10
	81.8° C	83.0° C	84.2° C	85.1° C
Eukarya	Well 4	Well 5	Well 6	Well 7
	78.2° C	79.3° C	80.5° C	81.8° C

Each of these samples contained 2 µl template DNA, 12.5 µl ReadyMix^{TM} Taq PCR Reaction mix, 8.5 µl dH_2O, and 1µl of each type of primer. The PCR cycles were the following as shown in Table 18.

Table 18 PCR Cycles for Gradient

95°C/2 min			
94°C/1 min	82°C/2 min	82°C/1 min	30 cycles
72°C/5 min			3 cycles

Three 1% agarose gels were prepared and 2 of the gels were run with the PCR products from the gradient. EtBr (2 µl) (10 µg/µl) and blue juice (2 µl) were added to each of the samples.

Once bands were obtained from these gels, they were isolated by being cut out with a sterile razor blade and placed in a spin column in a microcentrifuge tube. This was centrifuged for 10 minutes at 12,000 rpm. Two and a half volumes of ice cold 95% EtOH and one-half volume 7.5 M NH_4oAc were added to the remaining liquid and this was stored in the freezer overnight. The next day the tube was spun down for 20 minutes at 14,000 rpm. The supernatant was poured off and 20 µl DIUF water was added. This isolated DNA was then stored in the freezer at -20° C.

Cloning

Digest Reactions

Digest reactions were performed with primers from Table 12 using 3 µl PCR product (Eub), 5 µl 28i LITMUS vector, 2 µl Buffer E, and 1 µl *BamH* 1 enzyme. This mixture was

incubated at 37°C overnight. For plasmids with primers from Table 10, 3 µl PCR product was used with 5 µl 38i LITMUS vector, 2 µl Buffer E, 1 µl *BamH* 1 enzyme, 2 µl Buffer D, and 1 µl *Sal* 1 enzyme. This mixture was incubated at 37° C overnight. These enzymes were disabled by adding 10 µl phenol and 10 µl chloroform to each digest reaction. The tubes were spun in the tabletop centrifuge at 10,000 rpm for 10 minutes. The aqueous layer was removed from each of the tubes, placed into a new 0.5 ml tube, and 20 µl chloroform was added. This mixture was spun at 10,000 rpm for 10 minutes and the aqueous layer again removed and placed into a new 0.5 ml microcentrifuge tube. These digest reactions were then used for ligation reactions.

Ligation

Different ligation reactions were used. Ligation protocol #1 (Qiagen (Valencia, CA)) was followed according to the manufacturer's instructions. This involved 1 µl pDrive cloning vector, 4 µl of each of the isolated PCR products, and 5 µl Ligation Master Mix (2x). This was mixed gently and placed in the refrigerator for an hour and stored in the freezer overnight. Ligation protocol #2 (Promega Corporation (Madison, WI)) was used with a standard (Eub, Arch, Euk), a positive control, and a background control. For the standard reaction, 5 µl 2x Rapid Ligation Buffer was used with 1 µl pGEM®-T vector, 3 µl PCR product, and 1µl T4 DNA ligase (3 Weiss units/µl) for a total of 10 µl. For the positive control, 5 µl 2x Rapid Ligation Buffer, 1 µl pGEM®-T vector, 2 µl control insert DNA, 1 µl T4 DNA ligase, and 1 µl PCR water was used. For the background control, 5 µl 2x Rapid Ligation Buffer, 1 µl pGEM®-T vector, 1 µl T4 DNA ligase, and 3 µl PCR water was used. These five reactions were mixed by pipetting, incubated at room temperature for an hour, and stored in the freezer. For Ligation reaction #3, 4 µl of each PCR product was used (Eub, Arch, Euk) with 1 µl Salt Solution, and 1 µl TOPO

Vector (TOPO). This mixture was incubated at 22-23°C for 30 minutes. The reactions were placed in the freezer at -20° C. Ligation reaction #4 was also performed using Quick Stick Ligation Kit (Bioline). For this reaction, 4 µl digest product was used along with 10 µl DNA dilution buffer, 5 µl 4x Qs, and 1 µl QS DNA ligase. This mixture was incubated 5 minutes at room temperature and placed in the freezer at -20º C.

Transformation

Different transformation reactions were performed. Transformation reaction #1 involved adding 2 µl of each TOPO ligation reaction to a vial of Invitrogen Mach1-T1 chemically competent *E. coli* cells. These were incubated on ice for 30 minutes and heat shocked for 30 seconds in a heating block at 42°C. The vials were transferred to ice and 250 µl room temperature SOC medium was added to each vial. The tubes were shaken at 200 rpm in an incubator/shaker for 1 hour. After this incubation/shaking step, 50 µl of each transformation mixture was added to a prewarmed agar plate with 30 µl X-gal (20 µg/µl). These plates were placed in an incubator at 37° C overnight. A control reaction was done with 4 µl dH_2O, 1 µl Salt Solution, and 1 µl pCR4-TOPO. This mixture was incubated 5 minutes at room temperature then placed on ice and then 2 µl of this reaction was added to competent cells. Transformation reaction #2 was performed using three 1.5 ml tubes of Invitrogen chemically competent Mach1-T1 *E. coli* cells. These cells were thawed on ice for 5 minutes before 2 µl of standard ligation reaction was added to them. After they were flicked gently, 50 µl of the competent cells were transferred to tubes with ligation reactions. These tubes were gently flicked and placed on ice for 20 minutes. The cells were heat shocked for 47 seconds at 42° C and returned to ice for 15-20 minutes, and then 950 µl SOC medium (room temperature) was added to each tube. The tubes were placed for 1.5 hours in the shaking incubator at 37° C at 150 rpm. After incubation,

100 µl of each mixture (Eub, Arch, Euk) was added to an agar plate and the plates incubated overnight at 37° C. For Transformation reaction #3, one tube of competent cells (~200 µl) per phylogenetic domain (Eubacteria, Archaea, Eukarya) was thawed on ice 35 minutes. Next, 5 µl of each ligation reaction (Eub, Arch, Euk) was added to each of the tubes with the thawed cells and was mixed by flicking gently. Each of the tubes was heat shocked at 42° C for 30 seconds. The tubes were incubated on ice for 2 minutes and 250 µl room temperature SOC medium was added to each tube. Finally, 100 µl from each transformation mixture was placed on an agar plate with ampicillin and incubated at 37° C overnight. Transformation reaction #4 involved one vial of Invitrogen Chemically Competent *E. coli* cells being thawed on ice and 5 µl ligation reaction was added very carefully. This was mixed gently by flicking the tube and then incubated on ice 25 minutes. The mixture was heat shocked at 42° C for 30 seconds in a heating block. After returning the tube to ice, 250 µl SOC medium was added and the tube placed in the incubator/shaker at 37° C/200 rpm for 1 hour. After incubation/shaking, the mixture was placed on three prewarmed plates with 30 µl X-gal (20 µg/µl) each.

Preparation of Tubes for -80° C Freezer

To prepare tubes for storing clones at -80° C, LB broth powder (22.5 g) was added to 900 dH_2O and the mixture divided in half. One-tenth volume glycerol (50 ml) was added to the 450 ml volume. One ml was placed in each of one Fisherbrand Screwcap Microcentrifuge tubes (200) and these were autoclaved for 25 minutes. Once autoclaved, the tubes were stored at 4° C.

Preparation of Stock Plates with Clones

The white colonies that grew from LB transformation plates (Eubacterial, Archaeal, and Eukaryotic) were made into stock plates by streaking individual colonies on LB plates with

ampicillin (25 µg/µl) and X-gal (10 µg/µl). X-gal indicates whether the insert DNA (in this case it was the Eubacterial, Archaeal, or Eukaryotic 16s/18s ribosomal gene) was successfully ligated into the plasmid. The plasmid vectors used in this experiment contain genes for the enzyme β-galactosidase that metabolizes lactose. When lactose is present the bacteria will metabolize the sugar and produce a compound that combines with X-gal to produces a blue color—causing the colony to appear blue. However, if the gene sequence is successfully ligated into the plasmid, the gene for β-galactosidase is disrupted and the bacteria cannot metabolize lactose. This causes the colony to appear white. The stock colonies that were white were made into bacterial lawns that were scraped off using a sterile wire loop, given a number, and placed into 1.5 ml prepared tubes with LB + 10% glycerol and stored at -80° C. Transformation reactions with clones are shown in Table 19. Designation of clones is shown in Table 20.

Table 19 Numbers of Digest, Ligation, and Transformation Reactions Performed

Digest Reactions	Ligations	Transformations
5	12	21

Table 20 Designation of Clones.

	Clones Streaked for Stock	Put in -80° C for Storing	Minipreped	Check for Insert
Eubacteria	302	252	31	26
Archaea	395	17	62	17
Eukarya	132	140	28	14

Isolation of Plasmid DNA from Clones

To prepare bacterial clones for plasmid DNA isolation, the clones were briefly thawed. Bacteria were scraped from these tubes and placed onto warm, LB agar plates with ampicillin (25 µg/µl), 30 µl X-gal (10 µg/µl), and incubated at 37° C. An individual colony was taken from this plate with a sterile wire inoculation loop and placed into an 18 ml glass test tube containing 1 ml LB broth with 1 µl ampicillin (25 µg/µl). The bacteria were incubated overnight at 37° C in the shaking incubator (200 rpm). Plasmid DNA isolation (miniprep (Qiagen)) was performed according to the manufacturer's instructions. Eluted DNA was stored at -20º C.

All the miniprep reactions were combined according to phylogenetic domain (Eub, Arch, and Euk). They were purified using 2.5 volumes of 95% EtOH and 0.5 volume NH_4oAc. Volumes are displayed in Table 21. Once these solutions were added to each tube they were stored at -20° C about 4 hours and then they were taken out and centrifuged for 10 minutes at 10,000 rpm. The supernatants were then poured out and 100 µl Elution Buffer (from Qiagen Qiaprep® Spin Miniprep Kit) was added.

Table 21 Volumes of Liquid Recovered and Amounts of EtOH and NH_4oAc Added

	Volume Recovered	Volume EtOH Added	Volume NH_4oAc Added	Total Volume
EUB	300 µl	750 µl	150 µl	1200 µl
ARCH 1-8 (each)	400 µl	1000 µl	200 µl	1600 µl
EUK 1	400 µl	1000 µl	200 µl	1600 µl
EUK 2	300 µl	750 µl	150 µl	1200 µl

Insert Confirmation

To confirm that the insert was present in clones, a PCR reaction was run using 5 µl isolated plasmid DNA (from Qiagen miniprep), 1 µl of each FB and RS primer (Eubacterial with

BamH1 sites), 12.5 µl ReadyMix™ Taq PCR Reaction mix, and 5.5 µl water. This PCR reaction was run under the following conditions shown in Table 22. When the PCR reaction was complete, the products were checked on a 1% agarose gel.

Table 22 PCR Cycles for Insert Confirmation (Joplin 1998)

94°C/5 min			
94°C/1 min	50°C/2 min	65°C/2 min	5 cycles
94°C/1 min	55°C/1 min	72°C/1 min	35 cycles
72°C/5 min	4°C/park		

Control colonies also had to be obtained to show that the insert was not present in them. Individual blue colonies (insert not present) from Eubacterial, Archaeal, and Eukaryotic transformation reactions were obtained and grown in test tubes 1 ml LB broth with 1 µl ampicillin (25 µg/µl). These tubes were labeled and placed in the shaking incubator at 150 rpm at 37° C. Qiagen QIAprep® Spin Miniprep reactions were performed on these controls to isolate the plasmids. These plasmids were stored in 1.5 ml microcentrifuge tubes at -20° C. Table 23 shows the clones that the plasmid isolation reactions were performed on.

Table 23 Clones Checked for Insert

	Clones Checked
Eubacteria	Control, 002, 003, 006, 008-010, 012-015
Archaea	Control, 001, 003-015
Eukarya	Control, 001-010

Control *BamH* 1 Enzyme Digest

To determine whether the *BamH* 1 enzyme was working properly a control plasmid with an 800 bp insert that could be digested out with *BamH* 1 was obtained. This plasmid was pETt

and for this specific reaction 4 µl of the plasmid was used. For the digest reaction, 2 µl pETt plasmid was mixed with 0.4 µl *BamH* 1 enzyme, 0.8 µl Buffer E, and 4.8 µl PCR water to bring the total volume to 8 µl. This reaction was mixed and then placed in the incubator at 37° C overnight. The digested plasmid as well as 2 µl uncut plasmid were run on a 1% agarose gel. The samples were loaded into the gel (10 µl of ExACTGene ladder were used as a molecular weight marker) and the gel was run at 102 volts for 30-45 minutes. The gel was stained in 200 ml 1X TBE with 10 µl EtBr (10 µg/µl).

For the PCR reactions to check for the insert with the transformed bacterial colonies from above, four new 1% agarose gels were prepared. Archaeal and Eukaryotic clones were used and twenty-two 0.5 ml microcentrifuge tubes were obtained and labeled: for Archaea C (control), 1, 3-15 and for Eukarya: C (control), 1-10. For the PCR reactions, 5 µl of each clone plasmid DNA was placed into the correctly labeled tube and mixed with 12.5 µl ReadyMix™ Taq PCR Reaction mix, 1 µl of each correct FB and RS primer (Arch or Euk), and 5.5 µl PCR water. These reactions were mixed by pipetting and then placed into the Eppendorf thermocycler and run with the same cycles as Table 15. The samples were loaded into the 1% agarose gels. On two of the gels with the Archaeal samples, 10 µl of the ExACTGene DNA ladder was used. These gels were run at 102 volts for 45 minutes. On the other two gels with the Eukaryotic samples, 10 µl of *λDNA-HindIII/øx-HaeIII* (F-303XSD) DNA molecular weight marker was used. These gels were run at 104 volts for 30 minutes. The gels were stained in 200 ml 1X TBE buffer with 10 µl EtBr (10 µg/µl) for 30 minutes. They were viewed on the transilluminator and photographed.

Sequencing

Clones were selected to send off to the University of Tennessee, Knoxville for sequencing as shown in Table 24. They were wrapped in parafilm and sent overnight to the sequencing lab. The sequences were returned as an e-mail attachment and opened using the BioEdit program. Once it was established what the actual sequence was (Appendix C), it was placed into the BLAST website (NCBI), for identification.

Table 24 Clones Sent for Sequencing

Clone
Euk 001
Eub 001
Arch 001
Euk 001
Eub 002
Eub 003
Eub 007
Eub 020
Euk Control
Euk 009

CHAPTER 3

RESULTS

Identification of Organisms Cultured Directly from Roaches

Culturing of Organisms

Bacterial organisms were cultured both anaerobically and aerobically by directly smearing roach gut tissue and feces on agar plates. The aerobically cultured organisms were grown on both nutrient and blood agar plates in a candle jar that raised the CO_2 levels. The anaerobically cultured organisms were grown on both nutrient and blood agar in a glass chamber with a $NaBH_4$ gas pack that took the O_2 out of the air. Many colonies were present from these culture conditions. Colonies were present on all eight of the plates showing typical colony morphologies. In Figures 4A-D and 5A-D all eight of these plates are present with their bacterial colonies.

A B

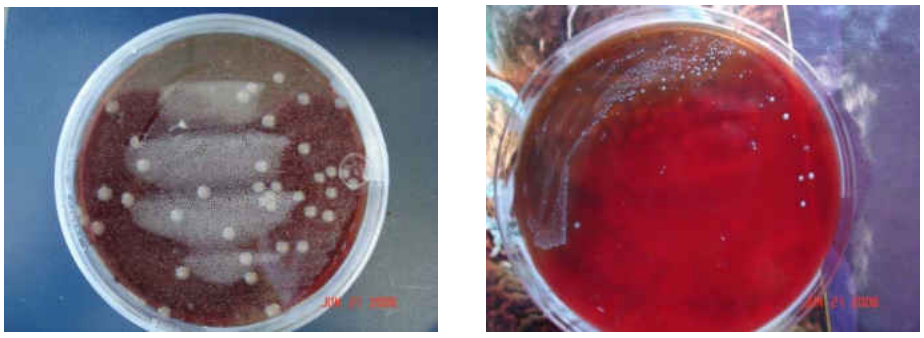
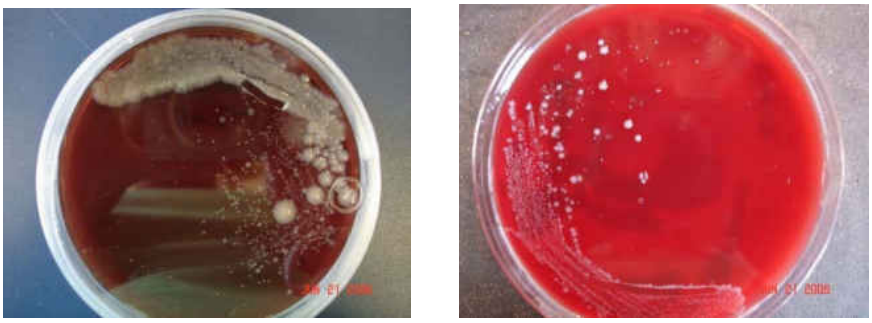


Figure 4A-B Bacterial Colonies Grown from Roach Feces. Figures A and B organisms were grown on blood agar. Figure A organisms were grown in the candle jar while Figure B organisms were grown in the anaerobic chamber.

C D

Figure 4C-D Bacterial Colonies Grown from Roach Feces. Figures C and D organisms were grown on nutrient agar. Figure C organisms were grown in the candle jar. Figure D organisms were grown in the anaerobic chamber.

A B



C D

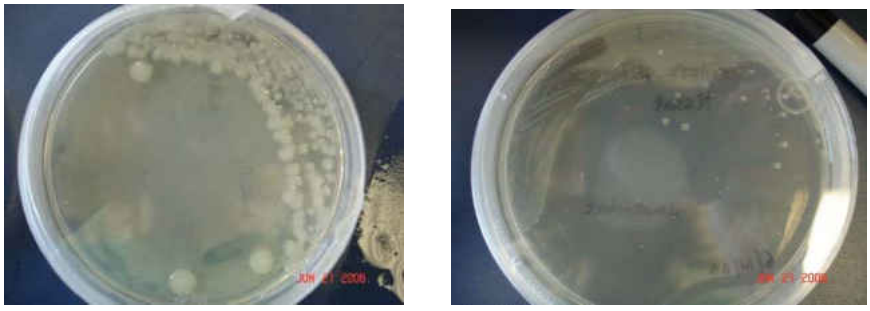


Figure 5 Bacterial Colonies Grown from Roach Gut Tissue. In Figures A and B organisms were grown on blood agar. In Figures C and D organisms were grown on nutrient agar. In Figures A and C organisms were grown in the candle jar. In Figures B and D organisms were grown in the anaerobic chamber.

Single Colony PCR

When single colonies were chosen off the plates for single colony PCR with Eubacterial primers bands were present in 8 out of 10 samples. Each of these bands represents the Eubacterial 16s ribosomal gene 1500 bp long. Figures 6A-B show PCR results from these reactions.

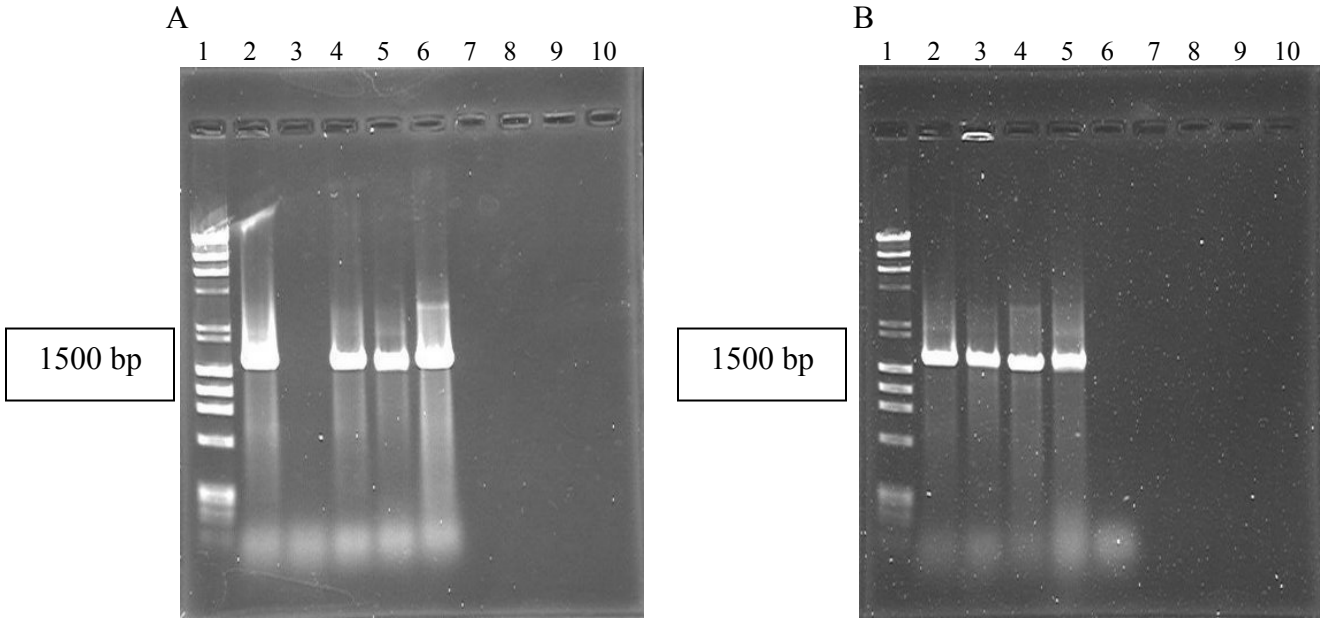


Figure 6 Single Colony PCR Results for Ten Bacterial Colonies. Figure A represents Samples 1-5. (Lanes 2-6). All positive reactions were 1500 bp. Figure B represents Samples 6-10 (Lanes 2-6). No results were seen with samples 2 and 10.

After purification of the DNA, reamplification results were seen for all samples (Figures 7A-B).

Chromosomal DNA Isolation

Chromosomal DNA isolated from bacteria scraped off the plates was very viscous. After being run on a gel, the anaerobic chromosomal DNA and candle jar chromosomal DNA was run

on a gel with the purified single colony PCR samples. The chromosomal DNA yielded results also shown in Figures 7A-B.

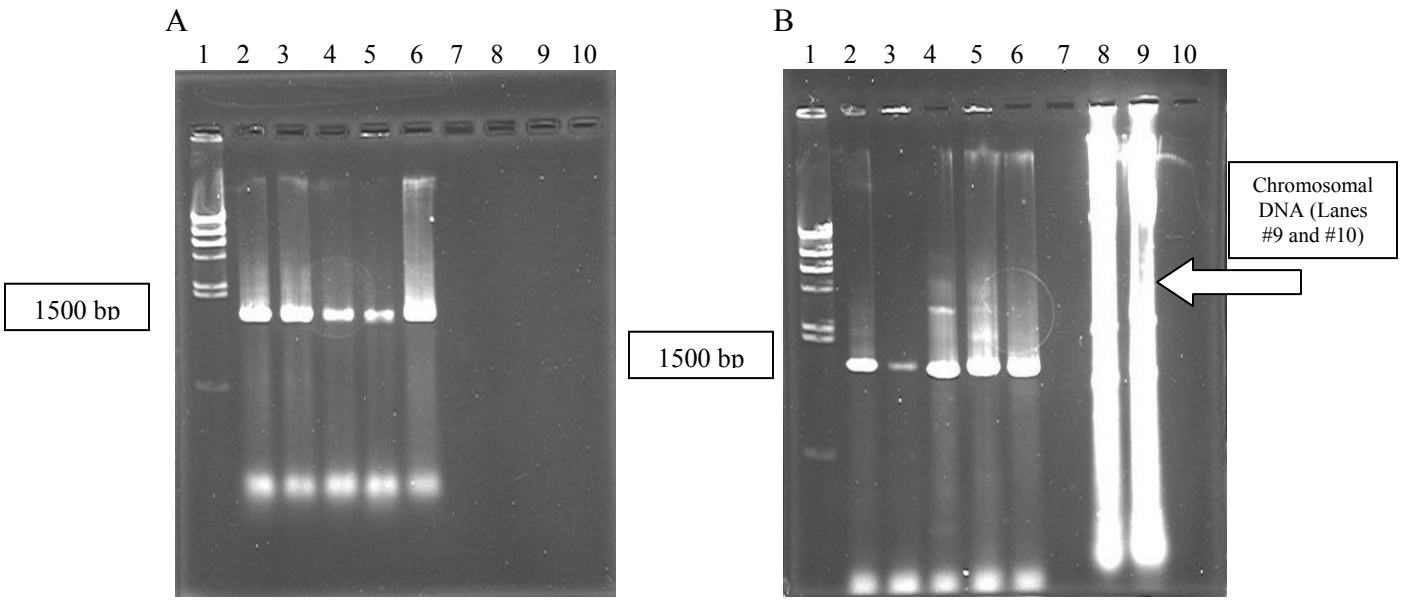


Figure 7 Backup PCR and Chromosomal DNA. Figures A and B represent backup PCR with the ten individual colonies. All ten samples showed bands 1500 bp which is the size of Eubacterial 16s ribosomal gene. In Figure A, Lanes 2-6 represent backup PCR samples 1-5. In Figure B, Lanes 2-6 represent backup PCR samples 6-10. Lanes 9 and 10 represent isolated chromosomal DNA from the bacteria scraped from the plates.

PCR from Combined Bacterial Colony DNA

The leftover chromosomal DNA (unspooled) was used for PCR amplification for all three phylogenetic domains (Eubacteria, Archaea, Eukarya), the chromosomal DNA was diluted 1/5000, and PCR reactions were run for all three phylogenetic domains. The resulting bands are shown in Figure 8. These products were purified using 95% EtOH and NH_4oAc.

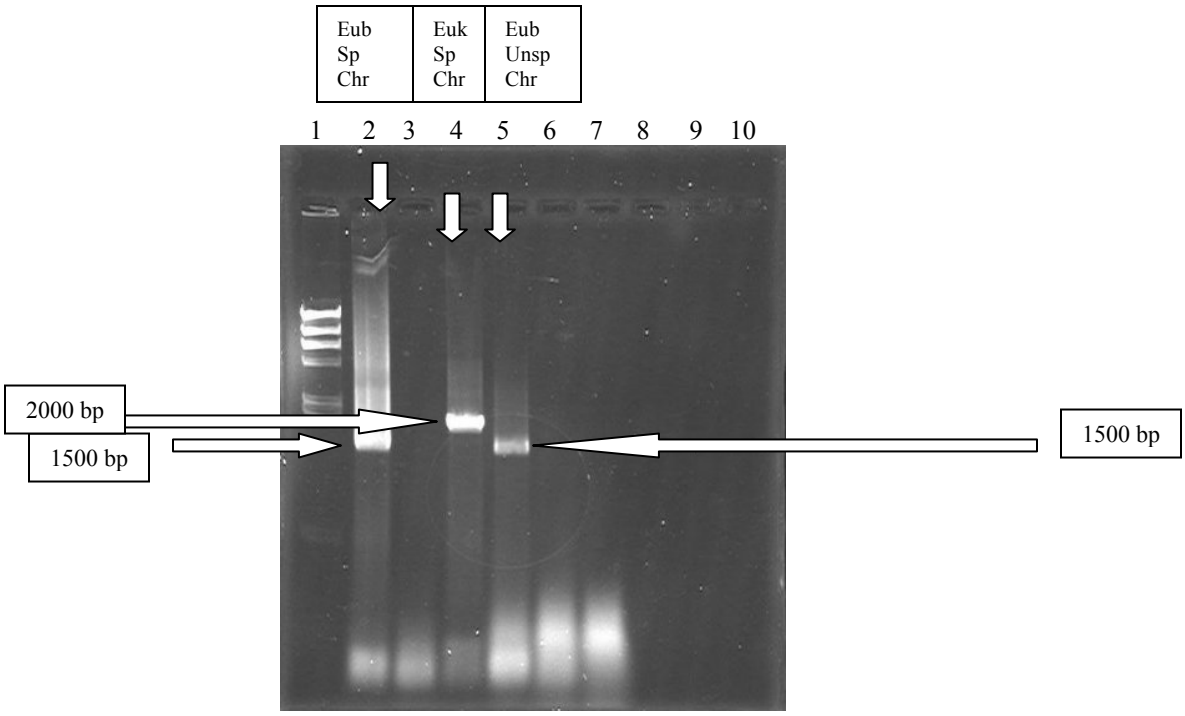


Figure 8 PCR Results from Bacterial DNA Isolation. The Eubacterial spooled chromosomal DNA was the streaked band in Lane #2 and it was 1500 bp, the Eubacterial unspooled chromosomal band was in Lane #5 and was a clean band around 1500 bp, and the Eukaryotic spooled chromosomal DNA band was in Lane #4 and was a clean band around 2000 bp. 2000 bp is the size of the Eukaryotic 18s ribosomal gene.

When the PCR products (spooled and unspoiled chromosomal DNA) were purified there were still bands present. Backup PCR reactions for the Eukaryotic samples (Euk Spooled Chr and Euk Unspooled Chr) yielded 2000 bp bands (size of Eukaryotic 18s ribosomal gene).

Ligation, Transformation, and Plasmid Isolation with PCR Products

There were few white colonies present on the plates that had not been diluted (10^0 concentration) after ligation and transformation. There were 32 white and blue colonies that were inoculated in the broth and only 11 that grew. These 11 included 2 Eubacterial and Eukaryotic controls (blue colonies that had been grown for a negative control), 6 Eubacterial rDNA samples, and 3 Eukaryotic rDNA samples.

Verification of Insert

After the double digest reaction (*BamH* 1 and *Sal* 1 enzymes) on the pGEM-T easy vector (Eukaryotic samples #1-3), there were 2000 bp bands present for all three (Figures 9A-B). This 2000 bp band represents the Eukaryotic 18s ribosomal gene. The PCR reaction for the Euk samples showed bands around 2000 bp in all four lanes including the control.

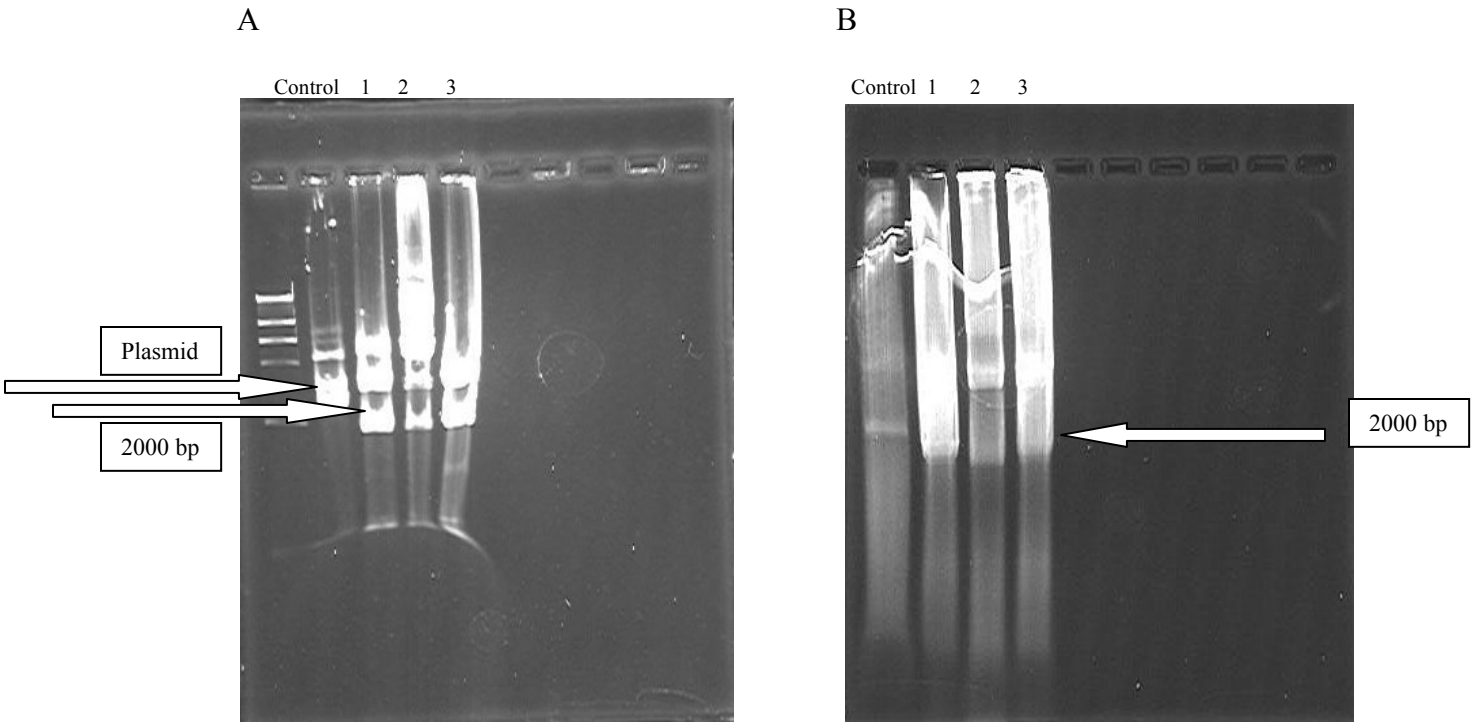


Figure 9 Results of Eukaryotic Digest and PCR. Figure A represents the digest using *BamH* 1 and *Sal* 1 enzymes. The last band present in Lanes #3-5 is around 2000 bp. The Control in Lane #2 does not show an insert. Figure B represents PCR products and every one of the samples contains a band around 2000 bp.

The digest with Eubacterial samples #1-10 showed single high molecular weight bands that suggested there was no 1500 bp (size of Eubacterial 16s ribosomal gene) insert present. The Eubacterial insert verification PCR reaction yielded 4 1500 bp bands; however, these results are inconclusive because there was no insert present in the digest reaction.

Sequencing and BLAST Results

Sequences obtained from UT Knoxville's sequencing lab are in Appendix C. BLAST results for Eubacterial samples #1-10 and Eukaryotic samples #1-3 are shown in Table 25. Phylogenetic trees created from BLAST are shown in Figures 10-16. The trees for Eubacterial Samples #2-3, 6, 9 and Eukaryotic Samples #2-3 are in Appendix D.

Table 25 BLAST Results for Samples

EUB 1	Closely related to genus *Enterococcus*
EUB 2	Closely related to genus *Enterococcus*
EUB 3	Closely related to genus *Enterococcus*
EUB 4	Closely related to genus *Klebsiella*
EUB 5	Member of genus *Pseudomonas*
EUB 6	Closely related to genus *Enterococcus*
EUB 7	Closely related to uncultured bacteria found in chemical treatment plants
EUB 8	Member of genus *Fusobacterium*
EUB 9	Closely related to the genus *Klebsiella*
EUB 10	Closely related to genus *Serratia*
EUK 1	Closely related to genus *Gromphadorhina**
EUK 2	Related to genus *Gromphadorhina**
EUK 3	Related to genus *Gromphadorhina**

* These three samples were actually roach DNA that had been cloned into a vector and sequenced.

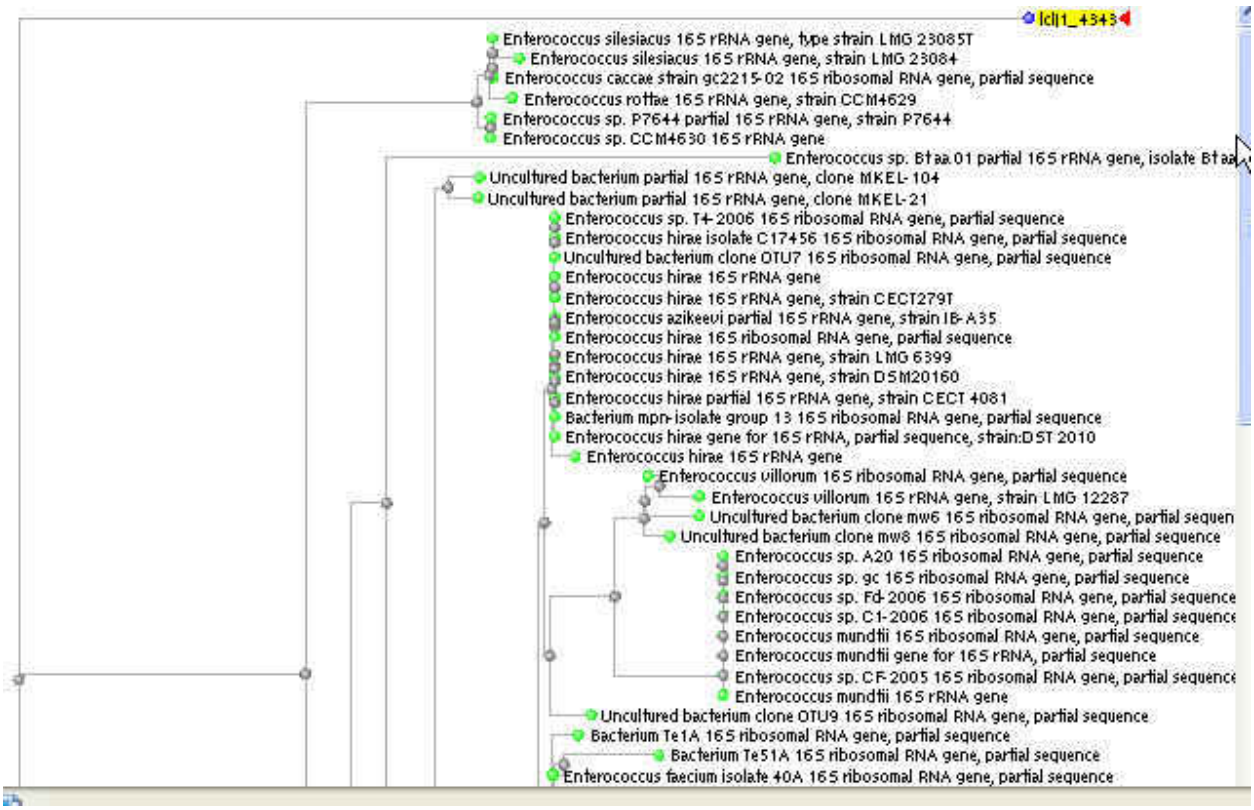


Figure 10 Eubacteria Sample #1 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_4343 represents the sample. This organism is closely related to the genus *Enterococcus*.

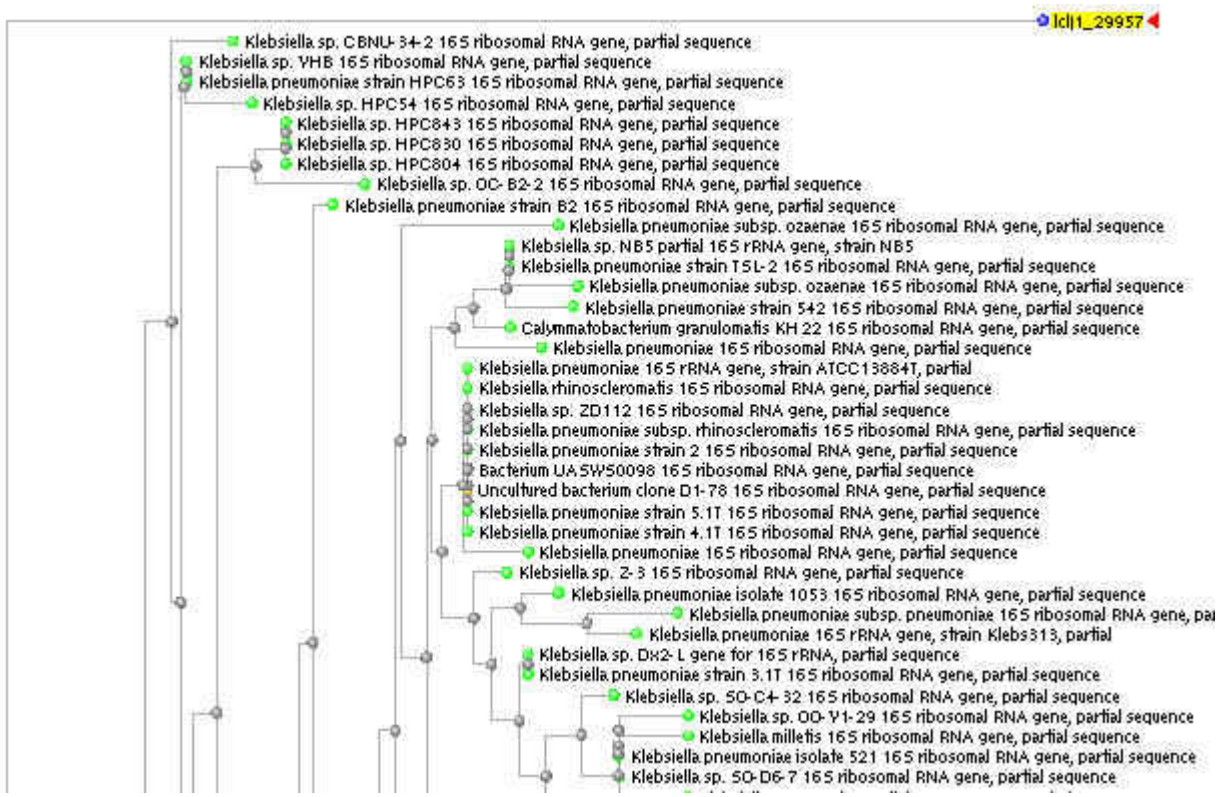


Figure 11 Eubacteria Sample #4 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_29957 represents the sample. This organism is closely related to the genus *Klebsiella*.

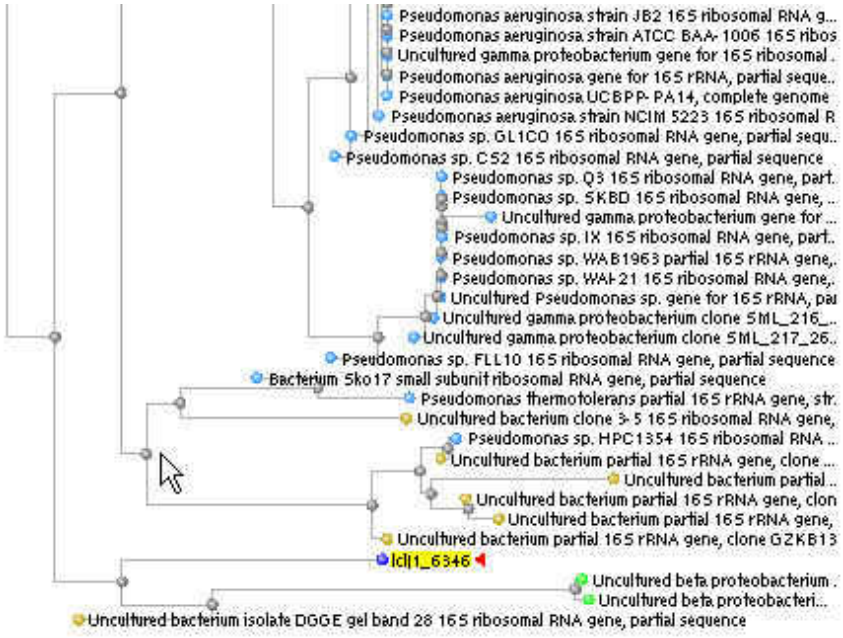


Figure 12 Eubacteria Sample #5 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_6346 represents the sample. This organism is a member of the genus *Pseudomonas*.

Figure 13 Eubacteria Sample #7 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_19068 represents the sample. This organism is an uncultured bacterium related to bacteria from a treatment plant.

Figure 14 Eubacteria Sample #8 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_26875 represents the sample. This organism is a member of the genus *Fusobacterium*.

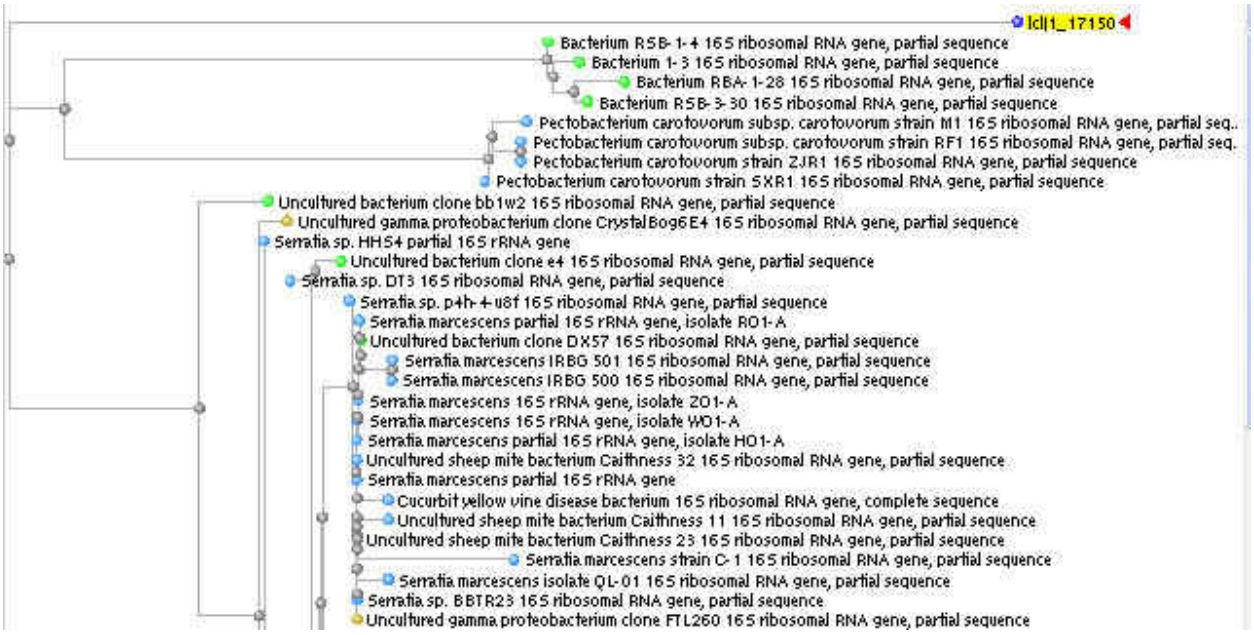


Figure 15 Eubacteria Sample #10 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_17150 represents the sample. This organism is closely related to the genus *Serratia*.

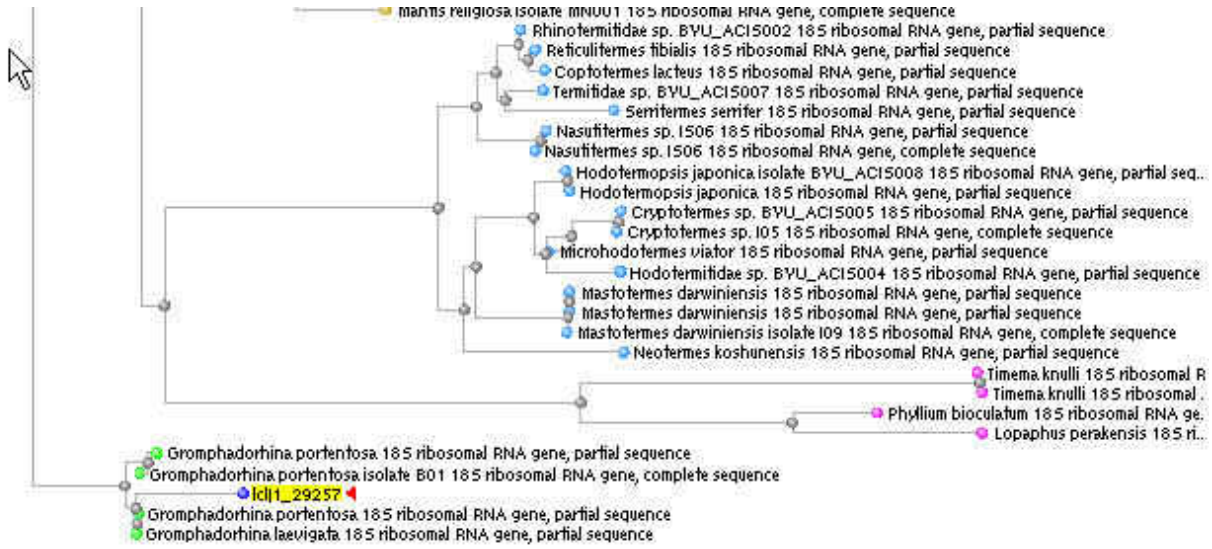


Figure 16 Eukaryotic Sample #1 Results of BLAST Phylogenetic Tree Analysis. The yellow highlighted mark labeled lcl|1_29257 represents the sample. The sample is roach DNA because the results here show that the sequence is within the genus *Gromphadorhina*.

ClustalW Results

Figure 17 shows Eubacteria sample #1 aligned with sequences it is related to.

```
E.durans              GGACGAACGCTGGCGGCGTGCCTAATACATGCA 52
E.faeciumhoneybee     GGACGAACGCTGGCGGCGTGCCTAATACATGCA 60
Unculturedmw8         GGACGAACGCTGGCGGCGTGCCTAATACATGCA 52
Unculturedmw6         GGACGAACGCTGGCGGCGTGCCTAATACATGCA 52
E.villorum            GGACGAACGCTGGCGGCGTGCCTAATACATGCA 40
E.ratti               -GACGAACGCTGGCGGCGTGCCTAATACATGCA 32
E.hiraeC17456         GGACGAACGCTGGCGGCGTGCCTAATACATGCA 33
E.azikeevi            GGACGAACGCTGGCGGCGTGCCTAATACATGCA 53
UnculturedOTU9        ----------------------TAATACATGCA 11
E.hirae               GGACGAACGCTGGCGGCGTGCCTAATACATGCA 42
E.faecium             -GACGAACGCTGGCGGCGTGCCTAATACATGCA 32
E.lactis              GGACGTACGCTGGCGGCGTGCCTAATACATGCA 47
E.sanguinicola        -GACGAACGCTGGCGGCGTGCCTAATACATGCA 32
Unculturedpeh55       -GACGAACGCTGGCGGCGTGCCTAATACATGCA 32
E.phoeniculoca        GGACGAACGCTGGCGGCGTGCCTAATACATGCA 39
Unculturedmkel        -GACGAACGCTGGCGGCGTGCCTAATACATGCA 32
1FB                   TCGANGACGCTGGCGGCGTGCCTAATACATGCA 33
E.species4fireant     CCCCAATCATC--TATCCCACCTTAGGCGGCTG 33
                                  * *  *

E.durans              AGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACG 112
E.faeciumhoneybee     AGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 120
Unculturedmw8         AGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 112
Unculturedmw6         AGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCATCGGAAAAAGAGGAGTGGCGAACG 112
E.villorum            AGTCGAACGCTTCTTTTTCCANCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 100
E.ratti               AGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACG 92
E.hiraeC17456         AGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 93
E.azikeevi            AGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 113
UnculturedOTU9        AGTCGAACGCCTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 71
E.hirae               AGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACG 102
E.faecium             AGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGGAAGAGTGGCGAACG 92
E.lactis              AGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACG 107
E.sanguinicola        AGTCGTACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAAAAGGAGTGGCGAACG 92
Unculturedpeh55       AGTCGTACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAAAAGGAGTGGCGAACG 92
E.phoeniculoca        AGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAAAAAGAGTGGCGGACG 99
Unculturedmkel        AGTCGAACGCTTCTTTTCCACCGAGTGCTTGCACTCATCGGGAAAGAGGAGTGGCGGACG 92
1FB                   AGTCGAACGCTTCTT-TCCCACCCCAGCTTGCTCCACCGGGA-AAGAAGAGTGGCGAACG 91
E.species4fireant     GCTCCAAAGGTTACCTCACCGACTTCGGGTGTTACA--AACTTTCGTGGTGTGACGGGCG 91
                        **  * *  *             *  **                * *** **  **

E.durans              G-GTGAGTAACACGTGGGTAACCTACCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 170
E.faeciumhoneybee     G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 178
Unculturedmw8         G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGAATAACACTTGGAAACAGGTG- 170
Unculturedmw6         G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 170
E.villorum            G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 158
E.ratti               G-GTGAGTAACACGTGGGTAACCTACCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 150
E.hiraeC17456         G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 151
E.azikeevi            G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 171
UnculturedOTU9        G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 129
E.hirae               G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 160
E.faecium             G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 150
E.lactis              G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 165
E.sanguinicola        G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 150
Unculturedpeh55       G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 150
E.phoeniculoca        G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 157
Unculturedmkel        G-GTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTG- 150
1FB                   G-GTGAGTAACACGTGGGTAACCTGCCCACCAGAGGGGGATAACACTTGGAAACAGGTG- 149
E.species4fireant     GTGTGTACAAGGCCCGGG-AACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGA 150
                      * ***   **  *  *** *** *   ** *     *   * *  *  *   **  * *
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Figure 17 Eubacteria Sample #1 ClustalW Alignment Results. Figure 17 continued.

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E.durans                --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 227
E.faeciumhoneybee       --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGAGT 235
Unculturedmw8           --CTAATACCGTATAA-TAATTAAAACCGCATGGTTTTAATTTGAAAGGCGCTTTACGGT 227
Unculturedmw6           --CTAATACCGTATAA-TAATTAAAACCGCATGGTTTTAATTTGAAAGGCGCTTTACGGT 227
E.villorum              --CTAATACCGTATAA-TAATTAAAACCGCATGGTTTTAATTTGAAAGGCGCTTTACGGT 215
E.ratti                 --CTAATACCGTATAA-CAATCAAAACCGCATGGTTTTGATTTGAAAGACGCTTTCGGGT 207
E.hiraeC17456           --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 208
E.azikeevi              --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 228
UnculturedOTU9          --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTCGATTTGAAAGGCGCTTTCGGGT 186
E.hirae                 --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 217
E.faecium               --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 207
E.lactis                --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 222
E.sanguinicola          --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 207
Unculturedpeh55         --CTAATACCGTATAA-CAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGT 207
E.phoeniculoca          --CTAATACCGTATAA-CAATTGGAACCGCATGGTTCTAATTTGAAAGACGCTTTCGGGT 214
Unculturedmkel          --CTAATACCGTATAA-CAATCGGAACCGCATGGTTCTGATTTGAAAGGCGCTTTCGGGT 207
1FB                     --CTAATACCGCATAA-TACATCGGATCTCATGGTCTGATGTTGAAAGGCGCTTTCGGGT 206
E.species4fireant       TTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCC-GAACTGAGAGAAGCTTTAAGAG 209
                          *      *   **          * *  *   *       *** **  *****

E.durans                GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 287
E.faeciumhoneybee       GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 295
Unculturedmw8           GCCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 287
Unculturedmw6           GCCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 287
E.villorum              GCCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 275
E.ratti                 GTCACTGATGGATGGACCTGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 267
E.hiraeC17456           GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 268
E.azikeevi              GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 288
UnculturedOTU9          GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 246
E.hirae                 GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 277
E.faecium               GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 267
E.lactis                GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 282
E.sanguinicola          GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 267
Unculturedpeh55         GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 267
E.phoeniculoca          GTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 274
Unculturedmkel          GTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 267
1FB                     GTCACTGGTGGATGGACCCGCGGTGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG 266
E.species4fireant       ATTA-----GCTTAGCCTCGCGACTTCGCGACTCGTTG-------TACTTCCCATTGTAG 257
                                 *  * * *  ***         ** ****        **  * **     *

E.durans                CTACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 347
E.faeciumhoneybee       CCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 355
Unculturedmw8           CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 347
Unculturedmw6           CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 347
E.villorum              CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 335
E.ratti                 CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 327
E.hiraeC17456           CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 328
E.azikeevi              CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 348
UnculturedOTU9          CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 306
E.hirae                 CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 337
E.faecium               CCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 327
E.lactis                CCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 342
E.sanguinicola          CCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 327
Unculturedpeh55         CCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 327
E.phoeniculoca          CCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA 334
Unculturedmkel          CAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA 327
1FB                     CGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA 326
E.species4fireant       C-ACG-TGTGTAGCCCA--------GGTCATAAGGGGCATGATGATTTGACGTCATCCCC 307
                        * *** *   ***** *        *** **  *   **    ** *      *  ***

E.durans                AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 407
E.faeciumhoneybee       AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 415
Unculturedmw8           AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 407
Unculturedmw6           AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 407

E.villorum              AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 395
E.ratti                 AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 387
E.hiraeC17456           AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 388
E.azikeevi              AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 408
UnculturedOTU9          AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 366
E.hirae                 AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 397
E.faecium               AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 387
E.lactis                AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 402
E.sanguinicola          AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 387
Unculturedpeh55         AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 387
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Figure 17 continued

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E.phoeniculoca          GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 394
Unculturedmkel          GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 387
1FB                     AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCA 386
E.species4fireant       ACCTTCCTCCGG--TTTGTCACCGGCAGTCTT--GCTA-----GAGTGCCCAACTTAATG 358
                          ** *  * **     *     ** * ****  ** *     **  * *  **  *

E.durans                ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 466
E.faeciumhoneybee       ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 474
Unculturedmw8           ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 466
Unculturedmw6           ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 466
E.villorum              ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 454
E.ratti                 ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 446
E.hiraeC17456           ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 447
E.azikeevi              ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 467
UnculturedOTU9          ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 425
E.hirae                 ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 456
E.faecium               ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 446
E.lactis                ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 461
E.sanguinicola          ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 446
Unculturedpeh55         ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGGAGAACAA 446
E.phoeniculoca          ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 453
Unculturedmkel          ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTTAGAGAAGAACAA 446
1FB                     ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT-GTCAGAGAAGAACAA 445
E.species4fireant       ATGGCAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC 418
                        * * *   * *    *   * *   *   * *    ***         *    *  ***

E.durans                GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 525
E.faeciumhoneybee       GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 533
Unculturedmw8           GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 525
Unculturedmw6           GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 525
E.villorum              GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 513
E.ratti                 GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 505
E.hiraeC17456           GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 506
E.azikeevi              GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 526
UnculturedOTU9          GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 484
E.hirae                 GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 515
E.faecium               GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 505
E.lactis                GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 520
E.sanguinicola          GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 505
Unculturedpeh55         GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 505
E.phoeniculoca          GGA-TGAGAGTAACTGTTCRTCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 512
Unculturedmkel          GGA-TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTAC 505
1FB                     GGG-TGAGAGTAACTGTTCATCCTTTGACGGTATCTGACCAGAAAGCCACGGCTAACTAC 504
E.species4fireant       GAGCTGACGACAACCATGCACCACCTGTCACTTTGCCCCCGAAGGGGAAGCTCTATCTCT 478
                        *   ***    ***  * *  *   ** *  * *    **  *  *  *   *** **

E.durans                GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 585
E.faeciumhoneybee       GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 593
Unculturedmw8           GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 585
Unculturedmw6           GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 585
E.villorum              GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 573
E.ratti                 GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 565
E.hiraeC17456           GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 566
E.azikeevi              GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 586
UnculturedOTU9          GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 544
E.hirae                 GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 575
E.faecium               GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 565
E.lactis                GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 580
E.sanguinicola          GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 565
Unculturedpeh55         GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 565
E.phoeniculoca          GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 572
Unculturedmkel          GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 565
1FB                     GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA 564
E.species4fireant       AGAGTGGT--CAAAGGATGTCAAGACCTGGTAAG-GTTCTTCGCGTTGCTTCGAATTAAA 535
                              *   *   **   *    *  *** *** *** * **  **  ** *   ****

E.durans                GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 642
E.faeciumhoneybee       GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 650
Unculturedmw8           GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 642
Unculturedmw6           GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAAG 642
E.villorum              GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 630
E.ratti                 GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 622
E.hiraeC17456           GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 623
E.azikeevi              GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 643
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Figure 17 continued

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UnculturedOTU9          GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 601
E.hirae                 GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 632
E.faecium               GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 622
E.lactis                GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 637
E.sanguinicola          GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 622
Unculturedpeh55         GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 622
E.phoeniculoca          GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 629
Unculturedmkel          GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 622
1FB                     GCGAGCGCAG-GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG--CTCAACCGGGGAGG 621
E.species4fireant       CCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGT 595
                         *    **    * * ** *   * *    ** **  **   *    ******  *

E.durans                GTCATTGGAAACTG 701
E.faeciumhoneybee       GTCATTGGAAACTG 709
Unculturedmw8           GTCATTGGAAACTG 701
Unculturedmw6           GTCATTGGAAACTG 701
E.villorum              GTCATTGGAAACTG 689
E.ratti                 GTCATTGGAAACTG 681
E.hiraeC17456           GTCATTGGAAACTG 682
E.azikeevi              GTCATTGGAAACTG 702
UnculturedOTU9          GTCATTGGAAACTG 660
E.hirae                 GTCATTGGAAACTG 691
E.faecium               GTCATTGGAAACTG 681
E.lactis                GTCATTGGAAACTG 696
E.sanguinicola          GTCATTGGAAACTG 681
Unculturedpeh55         GTCATTGGAAACTG 681
E.phoeniculoca          GTCATTGGAAACTG 688
Unculturedmkel          GTCATTGGAAACTG 681
1FB                     GTCATTGGAAACTG 635
E.species4fireant       CGTACTCCCCAGGC 655
                              **  *
```

Figure 17 Eubacteria Sample #1 ClustalW Alignment Results. The organisms seen here were chosen because they are different species that closely related to the sample. The other Eubacterial samples were compared with similar sequences as well.

DNA Isolation and Quantification from Roach Gut Tissue

DNA isolation #1 yielded no discernable DNA band in the gel electrophoresis. DNA Isolation #2, however, produced DNA with some degradation. DNA isolation #3 produced DNA; however, it was not very visible and 10 µl had to be used instead of 5 µl. DNA Isolation #4 yielded bright high molecular weight bands as shown in Figure 18. DNA Isolation #5 produced high molecular weight DNA with impurities present. Quantification results for DNA Isolations #2-4 are shown in Table 26.

Table 26 Values for DNA Isolation Quantifications

	260/280 Ratio	Normal Range
DNA Isolation #2	0.00185	1.5-2.0
DNA Isolation #3 (1)	0.97700	1.5-2.0
DNA Isolation #3 (2)	0.80000	1.5-2.0
DNA Isolation #4	1.66000	1.5-2.0

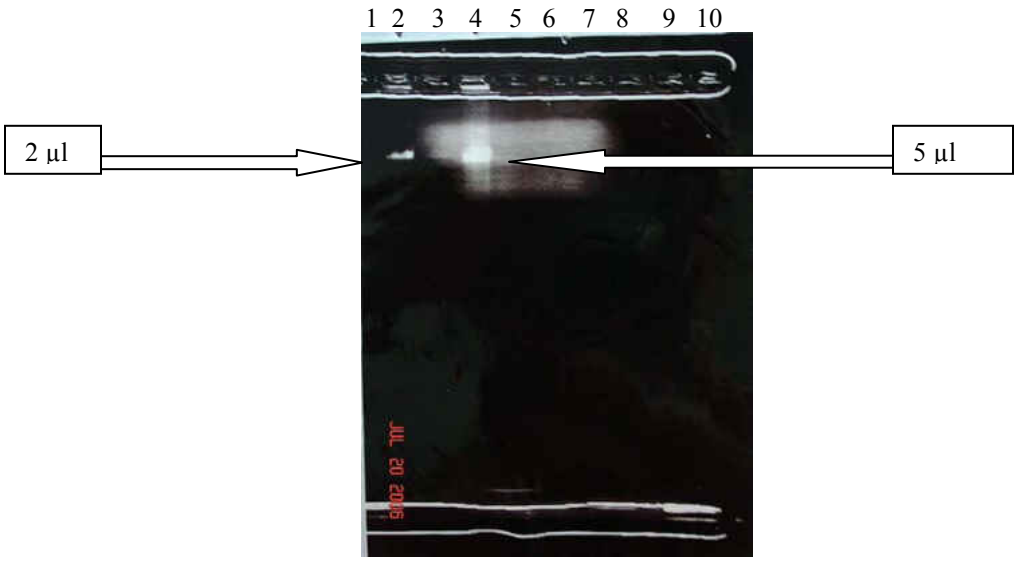

Figure 18 Total Genomic DNA Isolation #4. The first band represents 5 µl of high molecular weight DNA and the second band represents 10 µl of high molecular weight DNA.

When the total genomic *E. coli* DNA was extracted and run on the gel however, there was a high molecular weight band present. This is evident in Figure 19.

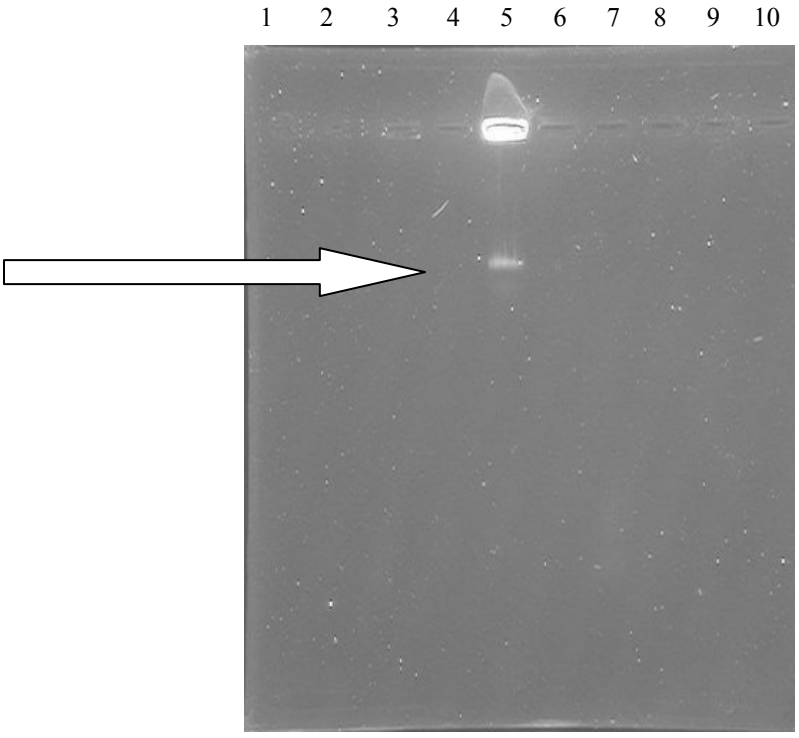

Figure 19 *E. coli* DNA Extraction Results. The extraction yielded in a large band which shows high molecular weight chromosomal DNA (Lane 5).

PCR Amplification

Of the 57 PCR reactions that were performed from DNA isolations #2-5, there were 20 positive results and 34 negative results as shown in Table 27. A positive reaction meant that there was a band of the correct size of the 16s/18s ribosomal gene for a particular phylogenetic domain (Eubacteria, Archaea, Eukarya).

Table 27 Results from All PCR Reactions

	PCR Reactions	% of Total Reactions
Positive Eubacteria	9	15.79
Positive Archaea	5	8.77
Positive Eukarya	6	10.53
Nothing	34	59.65
Other	3	5.26
Total Reactions	57	100

One of the most significant PCR results is shown in Figure 20. The Eubacterial bands were 1400-1500 bp (older primers had smaller bands)(represents 16s ribosomal gene size), the Archaeal bands were 975 bp (represents 16s ribosomal gene size) and were the same size and the Eukaryotic bands were around 1900 bp (represents 18s ribosomal gene size) and were roughly the same size. Following PCR reactions produced Eubacterial bands 1500 bp.

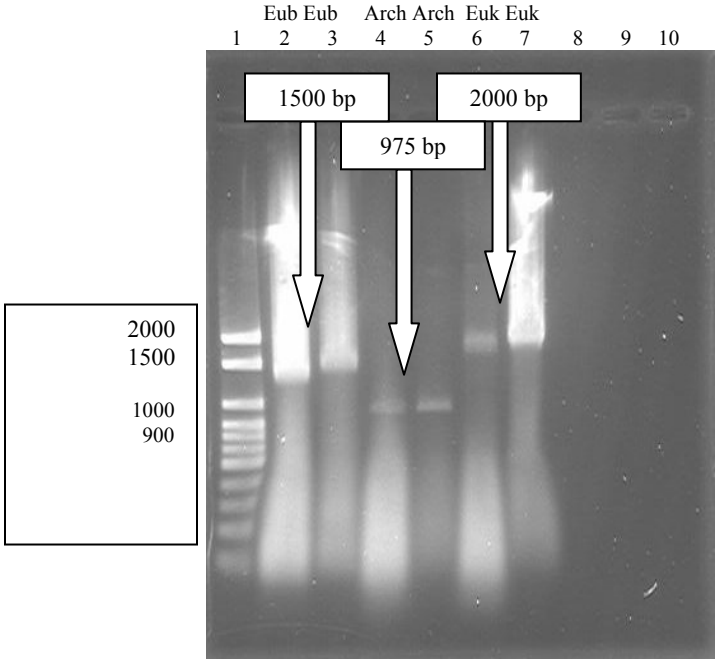

Figure 20 PCR Results from Old and New Eubacterial, Archaeal, and Eukaryotic Primers. The second, fourth, and sixth lane represents PCR bands using the old primers from Table 9 and the third, fifth, and seventh lanes represent PCR bands using the new primers from Table 10.

Cloning

The cloning reactions were done using digests, ligations, and transformations. The pDrive cloning vectors (Qiagen) yielded no colonies except on the Archaeal plates. The control reaction also yielded no results (no colonies grew on the plates). When the ligation and transformation reactions were redone using the pGEM®-T Vector (Promega) and Mach1-T1 chemically competent *E. coli* cells), white colonies were visible. However, these white colonies surrounded the present blue colonies and were much smaller, which indicates that these were satellite colonies. Satellites occur when the bacteria do not take up the plasmid with the ampicillin thus are unable to grow on the plates. However, the colonies that do grow produce penicillinase that breaks up the ampicillin and allows the bacteria without the plasmid to grow. They appear as smaller white colonies surrounding the blue ones. When the TOPO cloning kit (Invitrogen) as well as their transformation reaction with Mach1-T1 chemically competent *E. coli* cells was performed, nothing was present except one Eukaryotic colony. This colony was made into 8 stock colonies of which 7 grew.

Transformations using the 28i and 38i LITMUS vectors (New England Biolabs) and Quick Stick Ligation Kit (Bioline Inc), yielded many white colonies or colonies that contained the ribosomal gene insert. These isolates were streaked onto new plates and numbered to make them into stock colonies. The digest, ligation, and transformation reactions for the Archaea and Eukarya also produced white colonies. These were also streaked onto plates to make stock colonies. The transformation redone for the Archaea yielded mostly blue colonies with white satellite colonies.

When the transformation was performed after using phenol and chloroform extraction, white isolates were present. Results from this reaction were successful yielding white colonies.

Clones inoculated in LB broth for plasmid isolation, a significant number grew. The results of these digest/ligation/transformation/plasmid isolation reactions are displayed in Table 28.

Table 28 Results of Digest, Ligation, Transformation, and Plasmid Isolation Reactions

	Digests that Worked	Ligations that Worked	Transformations that Yielded Colonies	White Colonies Present	Number of Colonies Grown for Plasmid Isolation
Eubacteria	2	4	4	302	22
Archaea	4	7	7	395	81
Eukarya	2	3	4	132	26
Total Reactions	8	14	15	829	129

Insert Verification

Restriction digests (*BamH* 1 only and *BamH* 1 and *Sal* 1) done to determine whether inserts (16s/18s ribosomal genes) were present in the Eubacterial, Archaeal, and Eukaryotic clones were unsuccessful. However, some of the PCR reactions for the Eubacteria and Eukarya appeared to yield positive results (bands that were the right size for the ribosomal genes for these two phylogenetic domains) as shown in Figures 21A-B, 22C-D. Figures 22A-B represents PCR reactions for Archaeal insert verification. Results for the restriction digest and PCR reactions are shown in Table 29.

Table 29 Results of Restriction Digests and PCR Reactions for Insert Verification

	Positive Restriction Digest Samples	Positive PCR Reaction Samples
Eubacteria	31	0
Archaea	1	0
Eukarya	12	0

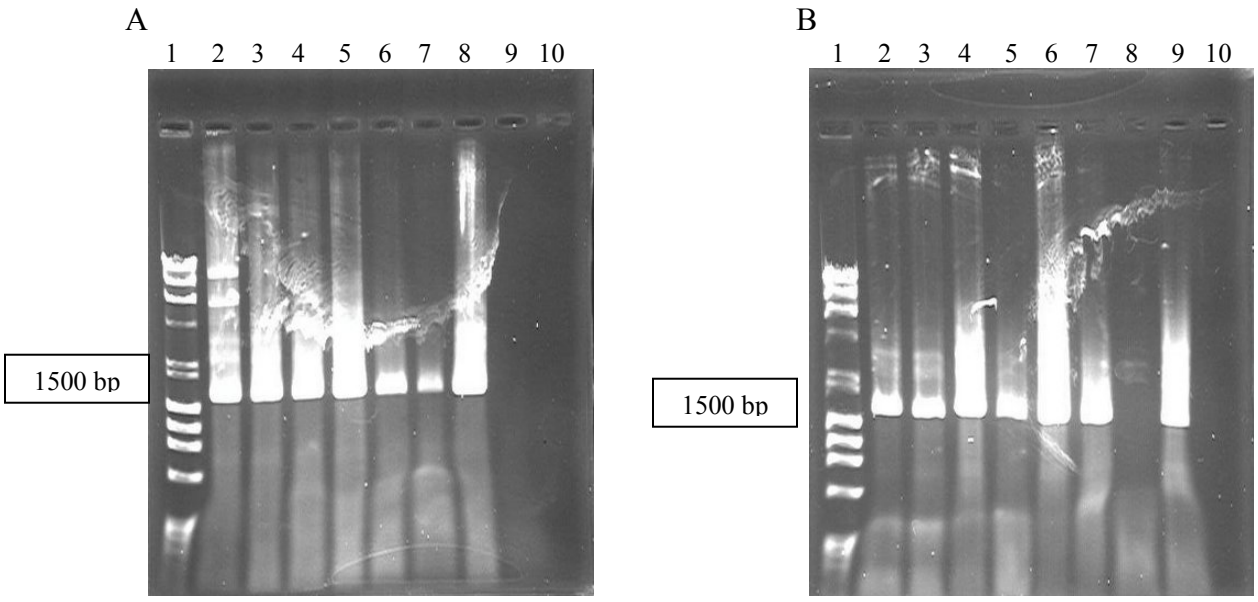

Figure 21 PCR Results for Eubacterial Insert Verification. Figure A represents Eubacterial Clones (PCR) Control, 004-008, 011, and 012 in lanes 2-9. Figure B represents Eubacterial Clones (PCR) 013-015, 020-022, 026, and 028 for insert verification (Lanes 2-9). The control represents a blue colony that was grown in broth, minipreped, and a PCR reaction done on this as well. Only Clone 026 in Figure B showed nothing (Lane 8).

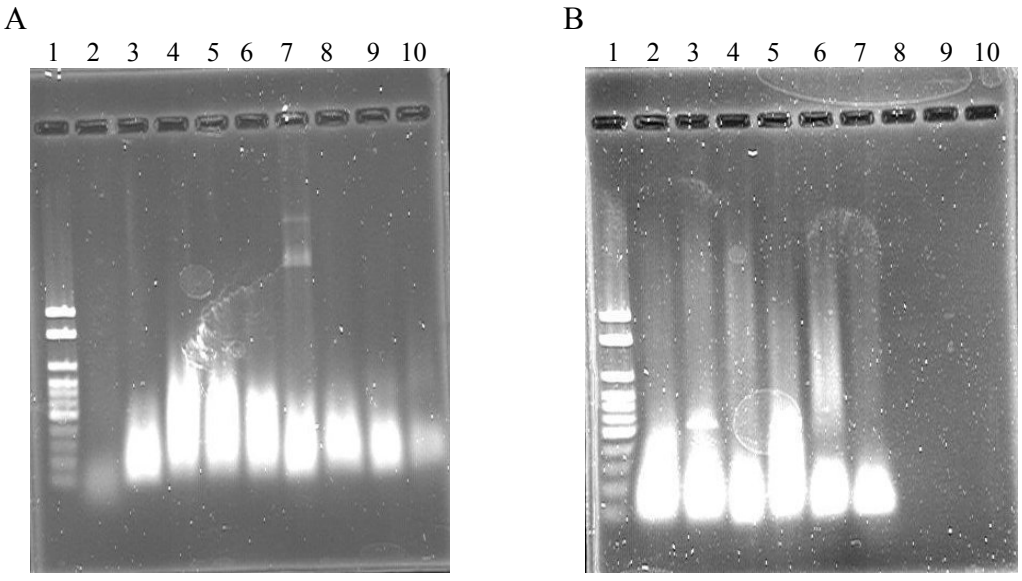

Figure 22A-B PCR Results for Archaeal and Eukaryotic Insert Verification. Figure A represents Archaeal Clones (PCR) Control 001, 003, 004, 005, 006, 007, and 008 in Lanes 2-10. Figure B represents Archaeal Clones (PCR) 009, 010, 011, 012, 014, and 015 in Lanes 2-7.

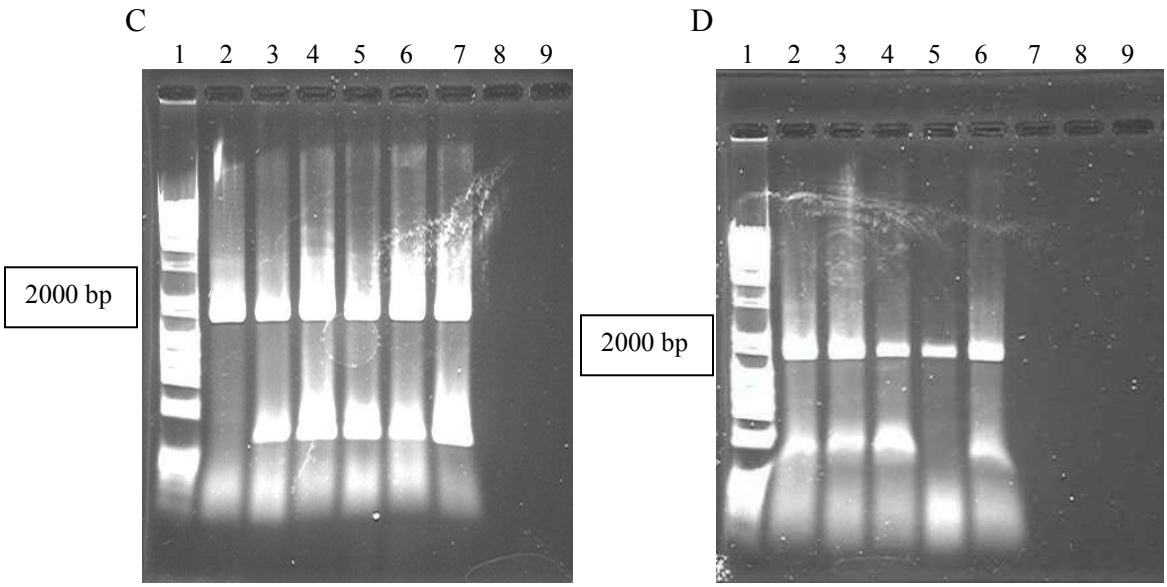

Figure 22C-D PCR Results for Archaeal and Eukaryotic Insert Verification. Figure C represents Eukaryotic Clones (PCR) Control, 001-005 in Lanes 2-7. Figure D represents Eukaryotic Clones (PCR) 006-010 in Lanes 2-6.

Control *BamH* 1 Reaction

When the *BamH1* enzyme was checked for activity using the pETt plasmid, results demonstrated that the enzyme was still active (Figure 23).

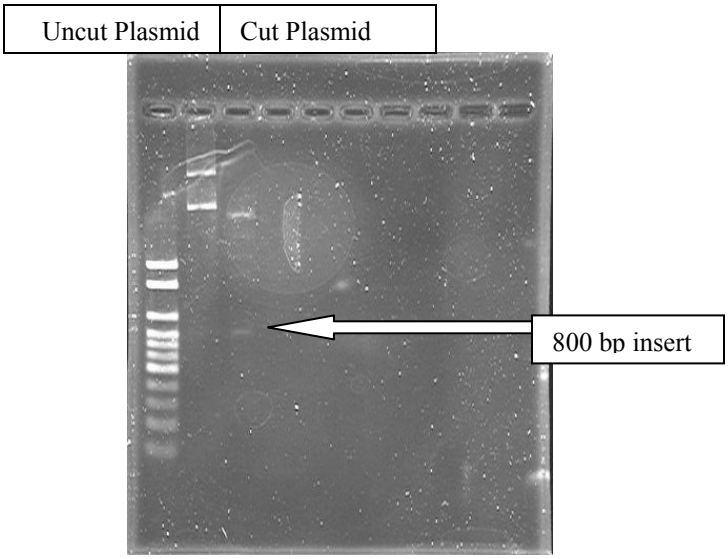

Figure 23 Digest Results from Control Reaction Checking *BamH* 1 Activity. Lane #3 shows 800 bp insert and high molecular weight plasmid.

Sequencing

Clones prepared and sent off to the University of Tennessee Knoxville's sequencing lab only yielded cloning vector sequences. No gene insert was present in any of the clones as shown in Table 30. These gene sequences can be found in Appendix C.

Table 30 Results of Clone Sequencing

Clone Name	Results
EUB 001	No Significant Similarity Found
EUB 002	No Significant Similarity Found
EUB 003	No Significant Similarity Found
EUB 007	No Significant Similarity Found
EUB 020	Cloning Vector
ARCH 001	No Significant Similarity Found
EUK Control	Cloning Vector
EUK 001 (1) T3	RAGE Cloning Vector pRIG20
EUK 001 (1) T7	No Significant Similarity Found
EUK 001 (2)	No Significant Similarity Found
EUK 009	No Significant Similarity Found

CHAPTER 4

DISCUSSION

Research Applications

The ecology of microorganisms in association with macro organisms is a largely unexplored area of research. Even today there are technical difficulties in identifying the diversity of these organisms and this hinders research. Studies have shown that it is possible to use molecular techniques to determine the diversity of life; however, this approach has also demonstrated that >90% of species are unknown. In addition, only preliminary studies have been published on the ecological interaction of this diversity of organisms. Interacting networks and web association is just beginning to be explained in ecology. The gut ecology would be an ideal model system since it has been shown that the alimentary canal of organisms harbors organisms comprising the three phylogenetic domains of life (Cruden and Markovetz 1987). The ecology of endosymbionts in the alimentary canal of insects is a model of micro ecology that could be explored. Research has previously demonstrated that there is apparently a vast diversity of both anaerobic and aerobic bacteria present in the gut of the American cockroach *P. americana* and the cave-dwelling roach *E. posticus*. Many of these are common identified species such as *Enterobacter agglomerans, Klebsiella oxytoca, Citrobacter freundii, Serratia* species, and *Streptococcus* species. Other organisms frequently isolated were C*lostridium sporogenes, Fusobacterium varium, Eubacterium moniliforme*, and *Peptococcus variabilis* (Cruden and Markovetz 1987). In addition to anaerobic and aerobic bacteria, protozoans harboring bacteria or having bacteria associated with them (usually these bacteria are methanogens) have been isolated. Spirochetes and nematodes have also been identified in the

hindgut (Cruden and Markovetz 1987, Gijzen and Barugahare 1992). The goal of my study was to identify and describe some of the typical endosymbionts involving bacterial species, archaeabacteria, and eukaryotes in the gut of *G. portentosa*.

Molecular techniques such as PCR and cloning have allowed scientists to investigate diversity of organisms without having to resort to traditional techniques such as cultivation on petri dishes or in broth. For example, scientists investigated the biodiversity of microbial eukaryotes in the Antarctic Ocean through the use of molecular techniques (PCR) that amplified SSU rNA sequences. Through the use of PCR amplification many new lineages of picoplankton and nanoplankton were identified (Moriera and López-García 2002). These PCR and cloning techniques were applied to total DNA extracted from *G. portentosa* gut in my experiment in hopes that a vast array of eukaryotes could be found and identified. The results shown in Figure 20 demonstrate that it is possible to amplify Eukaryotic DNA using domain-specific primers through PCR.

In addition, molecular techniques have also led researchers to discover that the majority of bacteria present in the guts of insects are γ-proteobacteria from the γ-3 subdivision of Proteobacteria. Five genomes from insect endosymbionts have been sequenced, allowing for better understanding of how symbiosis is established. These secondary bacteria can be found not only in the gut tissue but also in glands, body fluids, or in cells that surround other endosymbionts. These bacteria are primarily the results of multiple independent infections (Gil et al. 2004). It is possible that several of the organisms identified in this study such as had gained access to *G. portentosa's* alimentary canal through independent infections such as handling, food, and drinking water. The reasoning behind this is that when the organisms were identified, Eubacterial Samples 7 and 10 were organisms typically found in the environment and

would only have entered the roach through the environment. Bacterial organisms that were identified appeared to belong to these groups of γ-proteobacteria since they belonged to or were very closely related to the genera *Enterococcus, Fusobacterium, Klebsiella*, *Pseudomonas*, and *Serratia*.

Isolation and Identification of Microflora from Roach Gut and Feces

The bacterial colonies cultured from the extracted roach gut and feces were cultured both anaerobically (anaerobic chamber) and aerobically (candle jar). These traditional methods were used because the digest, ligation, and transformation reactions used previously did not appear to produce detectable results. BLAST results showed that the previous sequences using molecular techniques were inconclusive. Single colony PCR amplification however, yielded bright 1500 bp bands that represented the size of the Eubacterial 16s ribosomal gene. BLAST results from these PCR amplifications, and sequencing demonstrated that these sequences belonged to bacteria. Eight of the 10 of the samples were typical bacteria found within the gastrointestinal tract of the roach and belong to five major genera of enteric bacteria. The other two samples can be found in the environment (#7, 10). Eubacterial samples #1-3, and 6 were very closely related to the genus *Enterococcus*. Eubacterial sample #8 was closely related to the genus *Fusobacterium.* Eubacterial samples #4 and #9 were very closely related to the genus *Klebsiella*. Eubacterial sample #5 was very closely related to the genus *Pseudomonas*. Eubacterial sample # 10 was very closely related to the genus *Serratia*. Eubacterial sample #7 was closely related to uncultured bacterial clones found in soil samples, *Pseudomonas*, and a hypertrophic freshwater lake in China. The identity of this organism was unclear.

The genus *Enterococcus* comprises the group of gram-positive cocci (circular-shaped organisms) that often appear in pairs, alone, or in chains. These bacteria are facultatively anaerobic and can be found in soil, food, water, plants, animals, humans, birds, etc. When they inhabit an animal or a human, however, they inhabit the gastrointestinal or genital tract. When these organisms escape the gastrointestinal tract and inhabit other parts of the body they may cause bacteremias and urinary tract infections (Facklam et al. 1970). Four out of 10 of the identified bacterial organisms from the *G. portentosa* gut were very closely related to this genus of bacteria. These are what would be the expected bacteria or organisms that would normally be encountered in an ecological study like this one.

The genus *Fusobacterium* comprises a group of anaerobic, gram-negative bacilli that are typically found in the mouth, upper respiratory tract, and intestinal tract of healthy individuals (Jousimies-Somer et al. 1970). One of the 10 identified bacterial samples was very closely related to the genus *Fusobacterium*.

The genus *Klebsiella* is a group of nonmotile, rod-shaped, gram-negative organisms that contain a capsule. They are anaerobes that are primarily found in the gastrointestinal tract, skin, and pharynx of humans but also thrive in the gastrointestinal tract of other organisms as well. Klebsiellae are often responsible for opportunistic infections in the respiratory tract and may produce beta-lactamase which is an enzyme that degrades penicillin and other beta lactam antibacterial drugs (Umeh and Berkowitz 2006). Two of the 10 bacterial samples obtained through single colony PCR were very closely related to the *Klebsiella* genus.

The genus *Pseudomonas* is a group of aerobic non-spore forming, gram-negative slightly-curved rods. These organisms are mobile because of flagella that propel them. These particular organisms are generally found in most moist environments. These include soil, on plants (fruits

and vegetables), and in watery environments such as swimming pools, hot tubs, and contact lens solutions. *Pseudomonas* colonizes the gastrointestinal tract of normal individuals and also may colonize the throat, nasal mucosa, and other moist skin surfaces. These organisms can cause infections in immunocompromised individuals and those with cystic fibrosis (Kiska and Gilligan 1970). One of the 10 identified bacterial samples was very closely related to this genus.

The genus *Serratia* is a group of organisms that are gram-negative, facultative anaerobic, rods, or coccobacilli. These bacteria are widely spread throughout the environment and can cause infections in the gastrointestinal tract (Abbott 1970). Of the 10 bacterial samples, only one was very closely related to *Serratia*.

The three Eukaryotic samples obtained from cloning DNA from cultured organisms from the roach gut appeared to all be roach DNA. The BLAST results show that these samples are all closely related to the Madagascar hissing roach *Gromphadorhina portentosa* and *Gromphadorhina laevigata*.

Total DNA Extraction from Roach Tissue

Results from the DNA extractions indicate that it is in fact possible using extraction methods to extract total genomic DNA from *G. portentosa*. There were a lot of impurities present in these DNA samples, however, and the DNA was often degraded, thus there was not a good, clean band of DNA on the gels. The DNA should have been more carefully extracted by using phenol and chloroform one after another instead of together. This also indicates that a more efficient method of total DNA extraction is needed.

PCR Amplification

In was established through PCR in Figure 20 that there were organisms comprising all three phylogenetic domains present in the alimentary canal of *G. portentosa*. Eubacterial bands were 1500 bp (16s ribosomal gene), the Archaeal bands were 975 bp (16s ribosomal gene) and the Eukaryotic bands were 2000 bp (18s ribosomal gene). Each band present in the gel represents gene sequences from thousands if not millions of different organisms belonging to each of the three phylogenetic domains (Eubacteria, Archaea, and Eukarya), some of these which have not been identified yet. These results indicate that the roach gut could serve as a model ecosystem because it houses organisms comprising the three phylogenetic domains of life.

Cloning and Sequencing

The majority of problems, however, with this study were with the digest, ligation, and transformation steps. The problems arose when the purified PCR DNA was digested with the restriction enzymes *BamH* 1 and *Sal* 1 and ligated into a vector. One suitable reasoning is that the enzymes were not working as they should or the wrong buffers were used causing the inefficiency. During earlier digests with the Eubacterial samples, both enzymes (*BamH* 1 and *Sal* 1) were used along with their specific buffers (Buffers E and D). However, when these same enzymes were just with just Buffer E (*BamH* 1 buffer), the insert was digested out. This could be because previously there was no insert present in the plasmid, or the enzyme was more efficient with just Buffer E. More reasons for why the ligation step did not work could be because the vector and insert were in the wrong proportions thus causing insert not to get into the vector. Another possibility is that the DNA could have been chewed up or degraded and thus not be able to be ligated into a vector. The transformation reactions faced many problems as well.

There were a lot of satellite colonies resulting when a transformation was performed. Another problem that occurred with the transformation is that in several instances the cells were old and did not grow. It was determined that the transformation reactions were unsuccessful when digest and PCR reactions were done to confirm the presence of the insert. None of the clones showed an insert present and when the PCR amplification reactions were done, they showed bands for the controls in addition to the regular clones. The majority of these transformation reactions were unsuccessful and the results inconclusive.

The clones that were selected to send off for sequencing (Table 24) all showed undetectable results or showed a cloning vector when placed into the BLAST database for analysis (Table 25). This demonstrates that the gene insert was not present at all. All of these clones indicate inconclusive results and were not used for ClustalW analysis. The gene sequences that were successfully ligated into the vector showed up as roach DNA. Even though the samples were roach DNA they can still be considered part of the model ecosystem because the host plays a role in the relationship with the endosymbionts.

Future Research

Future investigation to obtain a larger amount of organisms would be to use more culturing techniques besides just the candle jar and anaerobic chamber with the gas pack. Other culturing media could be used as well such as McConkey agar and LB agar. The visible organisms could then be collected, the DNA isolated, and PCR reactions could be done using more efficient primers such as Eubacterial 63f and 1387r (Marchesi et al. 1998). Another method would be to perform single colony PCR on as many different colonies as could be

detected. The gene sequences obtained from these colonies could then be placed into BLAST and identified based on sequence similarity and then placed into ClustalW for further analysis.

Further applications with my research could be that once more of organisms present in the roach gut are identified, they could be studied in closer detail to understand their interactions with the host organism. Studies with *N. ovalis* and its indwelling methanogens showed that the protozoan had a major effect on *P. americana* metabolism, body weight, and methane production (Gijzen and Barugahare 1992). These protozoans and methanogens are involved in methane production, hindgut metabolism, insect body weight, and generation time. When cockroaches were raised free of *N. ovalis* insect generation time increased, adult body weight decreased, and there was no methane production (Gijzen and Barugahare 1992). The knowledge of these organisms will then give researchers a better understanding of insects and how they interact with the world around them as well as their importance to the ecosystem.

Another example interaction of host organism/bacterial symbiosis is in honeybees. Four of particular symbionts contain bacteriostatic effects against the honeybee pathogen *Paenibacillus larvae larvae*. A study was done that isolated bacterial species from 7- and 1-day-old honeybee larvae. Twenty-three isolates that inhibited the pathogen were cultured from the larvae. These isolates included ten Bacillus sp. with an additional seven showing conditional inhibition. Of these 17 isolates the seven conditional inhibition ones belonged to the *Bacillus cereus* group and of the other 10 eight of them were *B. cereus* and two were *B. fusiformis*. In addition, matches with *Brevibacillus formosus*, isolates tied to *Stenotrophomonas maltophilia* as well as two *Acinetobacter* isolates inhibited the pathogen as well (Evans and Armstrong 2006). Further research with *G. portentosa* endosymbionts could demonstrate that these organisms work together to prevent disease in the roach.

Once it is established how the endosymbionts interact with their hosts, scientists can investigate how the symbionts interact with each other. For example, research has shown that the enteric organism *Escherichia coli* is capable of forming a biofilm with the help of other bacteria. Non-adhering *E. coli* strains were grown in the presence of other adhering bacterial species and formed biofilms and co-adhesion mechanisms. Non-adhering *E. coli* strain PHL565 was mixed with *Pseudomonas putida* and showed an increase in biofilm production and adherence to glass. There was an increase in the biomass of cells by four fold and the composition of the *E. coli* PHL565 was roughly the same as the *E. coli/P. putida* composition from the previous experiment. These experiments demonstrate that bacteria unable to adhere to surfaces may be enabled by the presence of other bacterial species, thus showing the benefits of an isolated ecosystem (Castonguay et al. 2006). It is possible that microfloral organisms inhabiting the *G. portentosa* alimentary canal could benefit each other in a similar way or even performing the same function.

Molecular techniques allow the identification of organisms and view of them from an ecological perspective—the roach alimentary canal is considered an isolated ecological system. Results support the working hypothesis (stating that organisms comprising all three phylogenetic domains are present in the roach alimentary canal and these include known as well as unknown organisms) and enhance previous studies that have been done using traditional techniques (Cruden and Markovetz 1987). The purpose of this experiment was an ecological study to get an idea of the number of endosymbionts (from all three phylogenetic domains) in the alimentary canal of roaches using molecular techniques. Through further research and improved techniques, it may be possible to identify more specific organisms from all three phylogenetic domains and further increase knowledge of the world of microbiology.

REFERENCES

Abbott S. 1970. *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia*. In: Murray PR, editor in chief, Baron E, Pfaller M, Tenover F, Yolken R, editors. Manual of Clinical Microbiology. 7th ed. Washington (DC): ASM Press. p 475.

Castonguay MH, van der Schaaf S, Koester W, Krooneman J, van der Meer W, Harmsen H, and Landini P. 2006. Biofilm Formation by *Escherichia coli* is Stimulated by Synergistic Interactions and Co-Adhesion Mechanisms with Adherence-Proficient Bacteria. Research in Microbiology. 157: 471-478.

Clark D and Shanklin D. 1995. Madagascar Hissing Cockroaches. The University of Kentucky College of Agriculture. http://www.uky.edu/Ag/Entomology/entfacts/misc/ef014.htm. Last accessed July 25, 2006.

Cruden DL and Markovetz AJ. 1987. Microbial Ecology of the Cockroach Gut. Ann. Rev. Microbiol. 41:617-43.

Delong. 1992. Archaea in Coastal Marine Environments. Proceedings of the National Academy of Sciences. 89:5685-5689.

Diez B, Pedrós-Alió C, and Massana R. 2001. Study of Genetic Diversity of Eukaryotic Picoplankton in Different Oceanic Regions by Small Subunit rRNA Gene Cloning and Sequencing. Applied and Environmental Microbiology. 67(7):2932-2941.

Dojka MA., Harris JK, and Pace NR. 2000. Expanding the Known Diversity and Environmental Distribution of an Uncultured Phylogenetic Division of Bacteria. Applied and Environmental Microbiology. 66 (4):1617-1621.

Evans JD and Armstrong TN. 2006. Antagonistic Interactions Between Honey Bee Bacterial Symbionts and Implications for Disease. BioMed Central Ecology. 6:4-12.

Facklam RR, Sahm DF, Teixeira LM. 1970. *Enterococcus*. In: Murray PR, editor in chief, Baron E, Pfaller M, Tenover F, Yolken R, editors. Manual of Clinical Microbiology. 7th ed. Washington (DC): ASM Press. p 297-298.

Falkowski PG and de Vargas C. 2004. Shotgun Sequencing in the Sea:A Blast from the Past? Science. 304:58-60.

Gijzen H J, and Barugahare M. 1992. Contribution of Anaerobic Protozoa and Methanogens to Hindgut Metabolic Activities of the American Cockroach, *Periplaneta americana*. Applied and Environmental Microbiology. 58 (8): 2565-2570.

Gijzen H J, Broers CM, Barughare M, and Stumm CK. 1991. Methanogenic Bacteria as Endosymbionts of the Ciliate *Nyctotherus ovalis* in the Cockroach Hindgut. Applied and Environmental Microbiology. 57 (6): 1630-1634.

Gil R, Latorre A and Moya A. 2004. Bacterial Endosymbionts of Insects: Insights from Comparative Genomics. Environmental Microbiology. 6 (11): 1109-1122.

Joplin K. 1998. Lab Notes.

Jousimies-Somer HR, Summanen PH, Finegold SM. 1970. *Bacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, and Other Anaerobic Gram-Negative Rods and Cocci. In: Murray PR, editor in chief, Baron E, Pfaller M, Tenover F, Yolken R, editors. Manual of Clinical Microbiology. 7th ed. Washington (DC): ASM Press. p 690.

Keeton WT. 2003. The Scientific Method, Fish Health and Pfiesteria. University System of Maryland, Fish Disease Information. http://aquaticpath.umd.edu/fishhealth/koch.html. Last accessed July 25, 2006.

Kiska LK, Gilligan PH. 1970. *Pseudomonas*. In: Murray PR, editor in chief, Baron E, Pfaller M, Tenover F, Yolken R, editors. Manual of Clinical Microbiology. 7th ed. Washington (DC): ASM Press. p 517.

Lackey J. 2001. Contig. The Dictionary of Cell and Molecular Biology Third Edition. http://www.mblab.gla.ac.uk/~julian/Dict.html. Last accessed July 25, 2006.

Lawrence T. Scaffold Proteins. 2001. The Dictionary of Cell and Molecular Biology. Third Edition. http://www.mblab.gla.ac.uk/~julian/Dict.html. Last accessed July 25, 2006.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, and Wade WG. 1998. Applied and Environmental Microbiology. 64:795:799

Morell V. 1997. Microbiology's Scarred Revolutionary. Science. 276:699-702.

Moriera D and López-García P. 2002. The Molecular Ecology of Microbial Eukaryotes Unveils a Hidden World. Trends in Microbiology. 10 (1):31-38.

Pace NR. 1997. A Molecular View of Microbial Diversity and the Biosphere. Science. 276:734-739.

Trevors and Long. 1998. DNA Isolation Procedure. American Biology Teacher. 60:276-280.

Ueda T, Suga Y, and Matsuguchi T. 1995. Molecular Phylogenetic Analysis of a Soil Microbial Community in a Soybean Field. European Journal of Soil Science. 46:415-421.

Umeh O and Berkowitz LB. 2006. Klebsiella Infections. Emedicine. http://www.emedicine.com/med/byname/klebsiella- infections.htm. Last accessed September 2, 2006. 1-30.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, et al. 2004. Environmental Genome Shotgun Sequencing of the Sargasso Sea. Science. 304:66-74.

Woese C. 1987. Bacterial Evolution. Microbiol. Rev. 51 (2): 221-271. http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=373105&blobtype=pdf. Last accessed July 25, 2006.

APPENDICES

APPENDIX A: Abbreviations

amp ampicillin

ARCH Archaeal/Archaea

BHI brain heart infusion

BLAST Basic Local Alignment Search Tool

bp base pairs

dH_2O deionized water

DIUF deionized ultra filtered

DNA deoxyribonucleic acid

dNTP deoxyribonucleoside triphosphate

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EMBL-EBI European Molecular Biology Laboratory-European Bioinformatics Institute

EtBr ethidium bromide

EtOH ethanol

Eub Eubacterial/Eubacteria

Euk Eukaryotic/Eukarya

IPTG Isopropyl-β-D-thiogalactopyranosid

LB Luria-Bertani

M molar

mg milligram

ml milliliter

mM millimolar

$NaBH_4$ sodium borohydride

NCBI National Center for Biotechnology Information

NH_4oAc ammonium acetate

PCR polymerase chain reaction

rDNA ribosomal deoxyribonucleic acid

rpm rounds per minute

rRNA ribosomal ribonucleic acid

SDS sodium dodecyl sulfate

TBE tris-boric acid EDTA buffer

TE tris-EDTA buffer

µg microgram

µl microliter

µM micromolar

X-Gal 5-Bromo-4-Chloro-3-Indolyl-Beta-D-Galactopyranoside

APPENDIX B: Recipes

1% Agarose Gel

30 ml TBE Buffer

0.03 gram Agarose

Heat for 45 seconds in microwave

Allow to cool 20-30 minutes

Staining Medium

200 ml 1X TBE buffer

10 µl EtBr (10 µg/µl)

10X TBE Buffer

55 g boric acid

9.3 g EDTA

108 g Tris Base

1000 ml distilled water

1X TBE Buffer

100 ml 10X TBE buffer

900 ml distilled water

X-gal Preparation

5 ml N, N-dimethylformamide to bottle of X-gal

10X TE Buffer

108 g Tris Base

9.3 g EDTA

For 1X TE add 100 ml 10X TE to 900 ml dH_2O

LB Broth

25 grams LB powder

1000 ml dH_2O

Autoclaved for 20-25 minutes

Ampicillin (25 mg/ml or 25 µg/µl)

20 ml dH_2O

500 mg ampicillin salt solution

LB Plates with Ampicillin (25 µg/µl) /X-Gal (20 µg/µl)

40 grams Luria-Bertani powder

1000 ml distilled water

Autoclaved 20-25 minutes

Cooled for 45-60 minutes

Ampicillin 25 mg/ml (25 µg/µl) (1 µl per ml of liquid)

30-40 µl X-Gal (20 µg/µl)

Insect Saline

7.5 grams NaCl

0.21 gram $CaCl_2$

0.25 gram KCl

Add to 1000 ml distilled H_2O and autoclave 20 minutes

70% EtOH

70 ml 200 Proof (100% EtOH)

30 ml dH_2O

APPENDIX C: Gene Sequences from BLAST

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TCGANGACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTCCCACCCC
AGCTTGCTCCACCGGGAAAGAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACC
TGCCCACCAGAGGGGGATAACACTTGGAAACAGGTGCTAATACCGCATAATACATC
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NNAACCGGGAGGG

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AANCCA

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AAGCCAN

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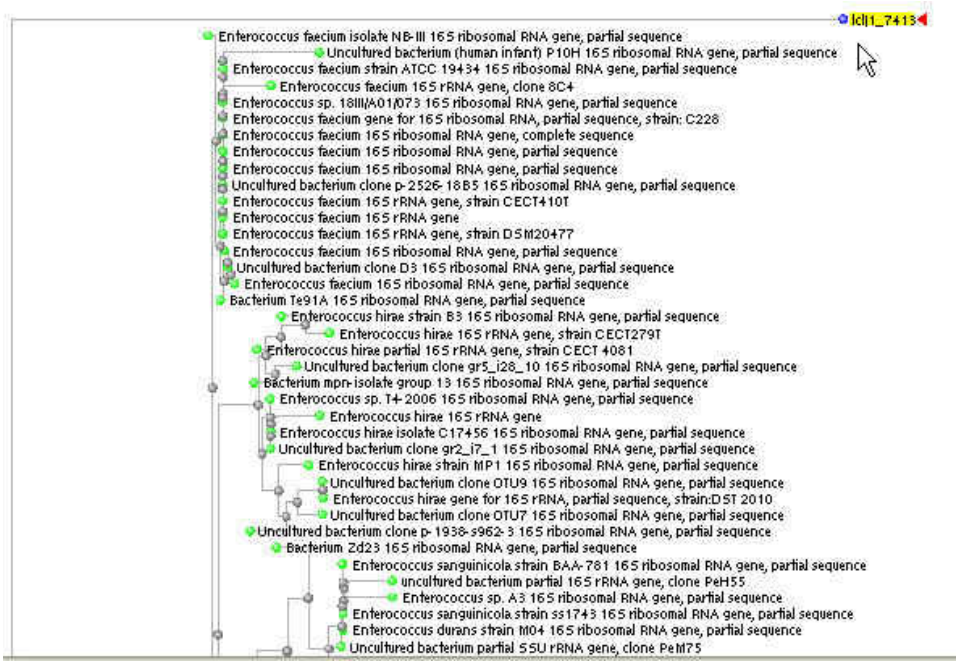
APPENDIX D: Phylogenetic Trees from BLAST

Eubacteria Sample #2 results of BLAST phylogenetic tree analysis



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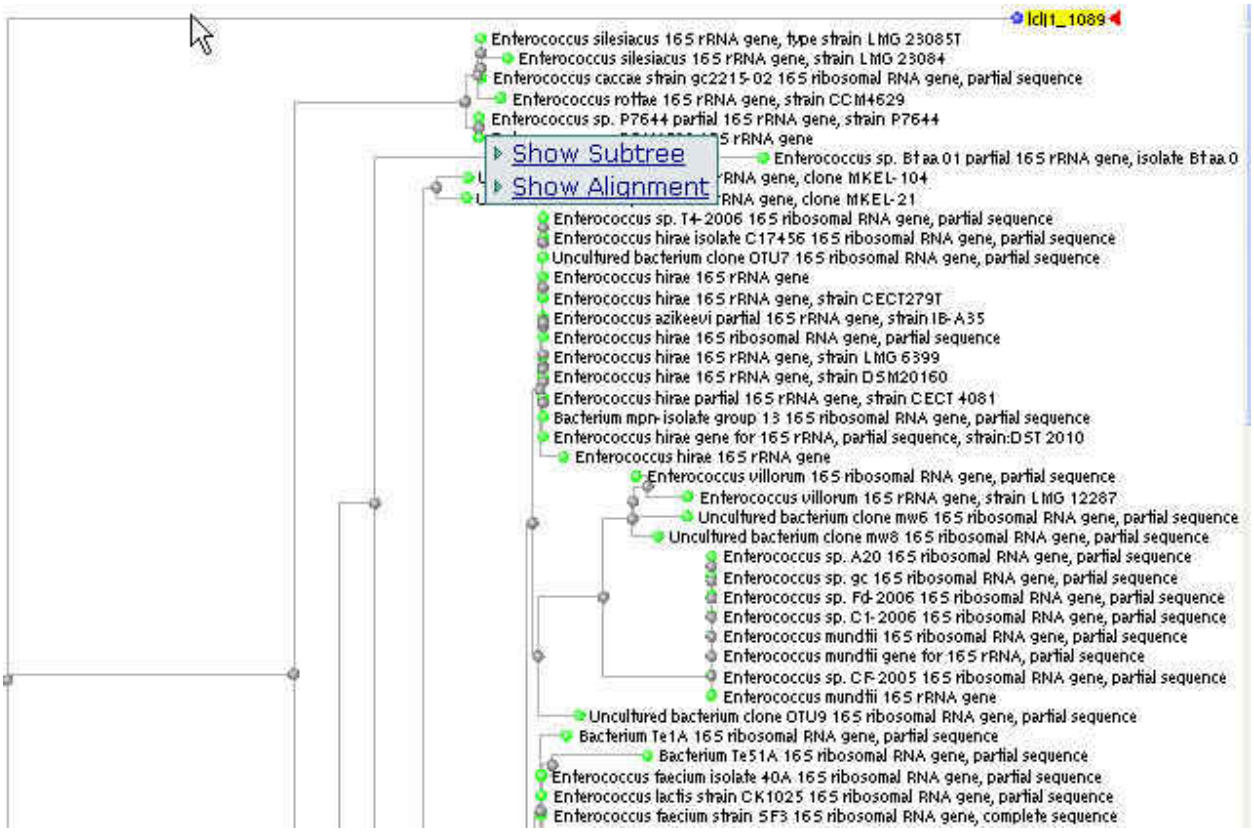
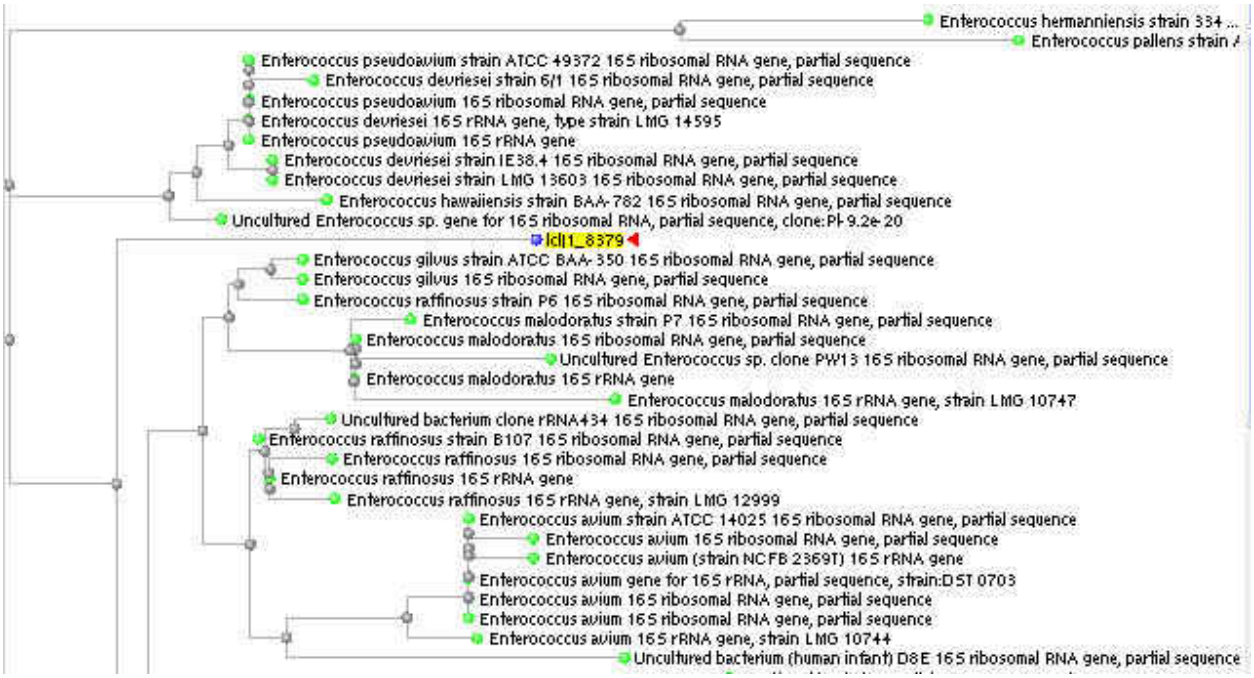


Eubacteria Sample #6 Results of BLAST Phylogenetic Tree Analysis



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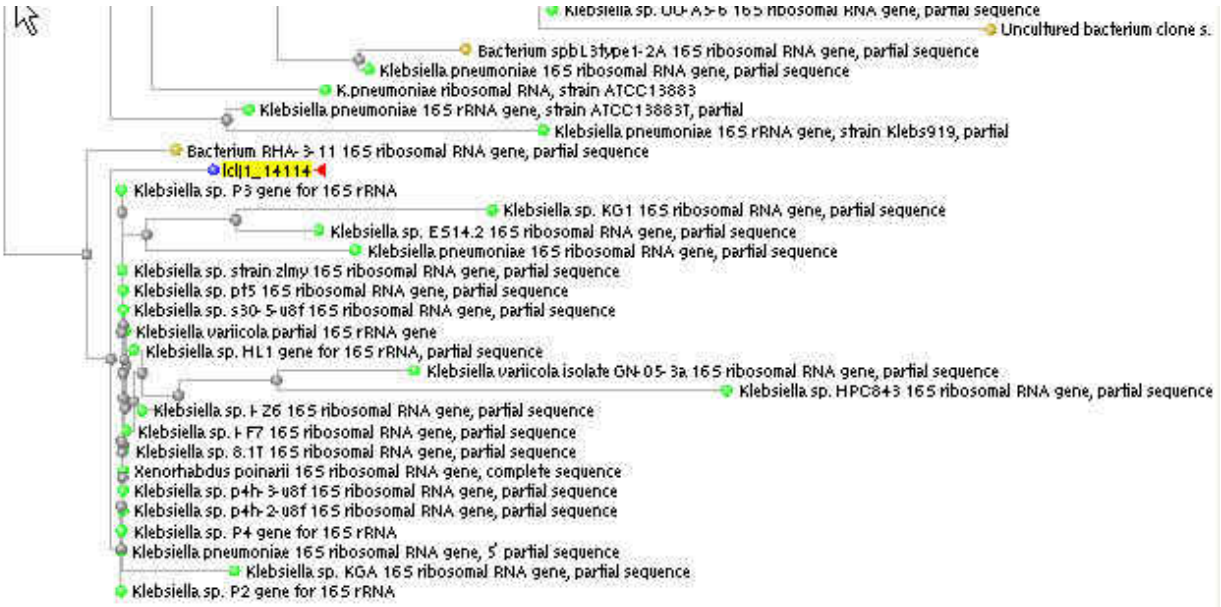
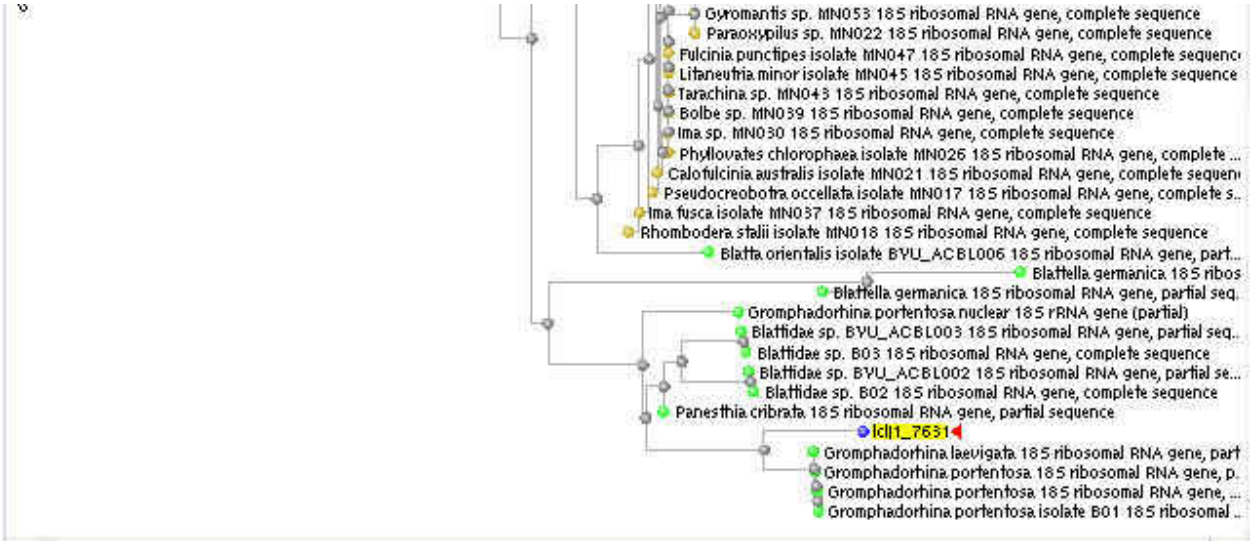


Eukarya Sample #2 results of BLAST phylogenetic tree analysis



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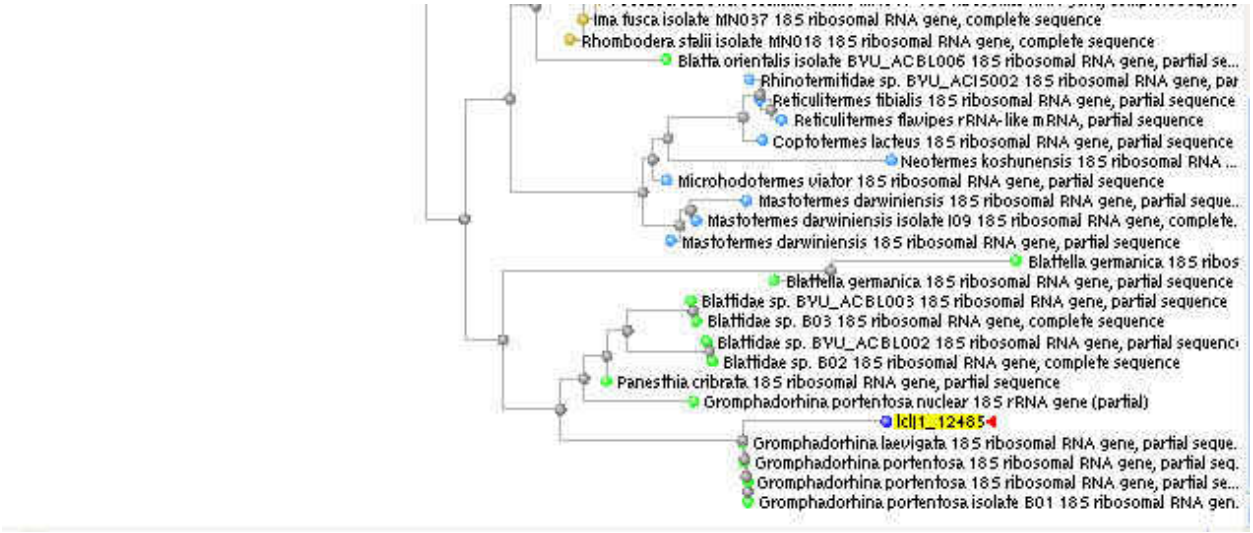

VITA

AMY RENÉE ROBERTSON

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Education:	Sullivan South High School, Kingsport, Tennessee, 1999 East Tennessee State University, Johnson City, Tennessee; Biology, B.S., 2004 East Tennessee State University, Johnson City, Tennessee; Biology, M.S., 2006
Professional Experience:	Graduate Assistant, East Tennessee State University, College of Arts and Sciences, 2004-2006
Publications:	None
Honors and Awards:	Search Committee for New Department Chair, 2005-2006 Academic Year The Chancellor's List, 2006 William Harvey Fraley and Nina M. Fraley Research Award, 2006