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POTENTIAL VIRAL SYMBIONT IN LEAF CUTTER ANT SYSTEM

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ABSTRACT

The ancient Attini ant symbiotic system, also known as the leaf-cutter ant system, is a textbook example of symbiosis. It consists of the Attini ant farmers that feed on the special mycelia structure, gongylidia, that is a part of their *Leucoagaricus gongylophorus* crop. The system is also host to *Escovopsis weberi*, a parasitic pathogenic fungus that is kept at bay by a bacterium utilized by the ants (Currie, 2001). Living among and within the three major players, there are many microorganisms that aid in the upkeep of the system that are continuously being discovered as knowledge of the system expands. *Leucoagaricus gongylophorus* is predicted to be host to a potential viral symbiont, likely a totivirus. The potential for a viral symbiont could expand the knowledge of mutualist viral symbionts to a multipartite symbiotic system. The study is conducted through dsRNA extraction from *L. gongylophorus* samples, random priming cloning, and RT-PCR. Further analysis through cloning and sequencing will provide the information needed to confirm the identity of the putative viral symbiont.

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Introduction

Symbiosis within the Attini System

Symbiotic relationships are widespread throughout nature. Many of these relationships have existed for as long as the organisms themselves, drawing questions as to their evolutionary history and how two very different organisms came together to exist as one. One of the most iconic examples of symbiosis is the Attini leaf-cutter ant system. This relationship consists of leaf-cutter ant farmers who cultivate the *Leucoagaricus gongylophorus* fungi and protect their crop through filamentous bacteria that suppress the *Escovopsis weberi* fungal pathogen (Currie, 2001). The relationship between ant and fungus is integral to the maintenance of the system, but not the only relationship that keeps the multipartite symbiosis alive. When studying symbiotic relationships, it is important to view all aspects with more than just a bilateral approach. From studying the Attini system from all angles, there is evidence of a new symbiont within the system, a totivirus. This virus is hosted by the *L. gongylophorus* fungus and was observed after dsRNA purification (unpublished data).

Symbiosis was first defined by Albert Bernhard Frank, and later by Anton de Bary as two entities that live on or in one another. They must be dissimilar and they must be in close physical contact (Roossinck, 2005). Within the Attini system, there are mutualists and antagonists. Symbiosis operates as a continuous gradient. It scales from high and low pathogenicity to commensal relationships, to mutual relationships that benefit both organisms. Typically, in symbiotic relationships, a virus is viewed as a parasite within its host. Although the virus is a

parasite, it cannot always be classified as antagonistic. Contrary to this view, some viruses are mutualists, essential to their host's life providing beneficial and necessary functions through their genome. It is hypothesized that the virus within *L. gongylophorus* aids in maintaining its mutualism with leaf-cutter ants of the Attini tribe by affecting the production of gongylidia.

Summary of the Attini System

Fungal agriculture has been a way of life for ants for over 50 million years with over 200 species deriving from the Attini tribe. As ant species evolved, they cultivated different distinct lineages of fungi all derived from the family Lepiotaceae (Currie, Mueller, *et al.*, 1999). The *Acromyrmex* and *Atta* ant genera exhibit maintenance of the most complex fungiculture with multifaceted colonies that can survive up to ten years.

The system begins with an ant queen who transfers a sample of the fungus from her maternal colony to a new location for her founding colony to proliferate. The queen has an opening called an infrabuccal pocket within her mouth in which she stores a small sample of the fungal cultivar (Currie, 2001). After the queen has been inseminated, she tends the fungus underground or sheltered within leaf litter, rocks, or logs. In the adult stage, workers tend to the fungus, but before her progeny reach maturity, the queen tends to the fungus through fecal fluids manure or substrate such as leaf litter (Currie, 2001). Within the *Acromyrmex* and *Atta* genera specifically, queens tend to their sample cultivars underground in a claustral chamber. The queen feeds the fungus with her own fecal material. Once the workers have reached maturity, they begin to collect leaf litter to commence the cultivation of their farm (Currie, Mueller, *et al.*, 1999).

The fungus produces gongylidia, a special mycelia structure, that the queen and larvae rely on as their sole food source. The gongylidia are hyphal swellings rich in carbohydrates and lipids ideal for larval growth. Besides gongylidia, the workers rely on different food sources such as dead vegetative matter, fresh leaves, and flowers (Miyashira, *et al.*, 2010).

This obligate mutualistic relationship is threatened by microbial pathogens and microorganisms that invade the leaf litter communities. These pathogens jeopardize the ants' most important food source as they compete for living space and nutrients within the soil (Schoenian, *et al.*, 2011). Attini ants have many inherent features that protect their fungal gardens from invading organisms. One quality is the coating of an actinomycete symbiont known as *Pseudonocardia* that has been found on their cuticle. It has antibiotic properties and has been proven adept at countering *E. weberi*, the fungus-growing ants' principal threat (Currie, Scott, *et al.*, 1999). In addition to their actinomycete symbiont, Attini ants also have been found to remove any invading species through licking their gardens and weeding through mastication. This can also be used to remove any infected fungal crops (Currie, 2001).

The *E. weberi* pathogen is an ascomycete fungus commonly associated with leaf-cutter ant gardens. As they have not yet been found outside of ant fungal gardens, they are assumed to have an obligate relationship with this symbiotic system. When *E. weberi* infects a healthy garden it completely decimates the crop within a few days' time. *E. weberi* is a necrotroph that kills the fungal crop to gain nutrients. The pathogen only relies on the cultivated fungus for nutrients and not the leaf litter or ant waste (Reynolds and Currie, 2004).

The organisms described play major roles in the symbiotic system of the *L. gongylophorus* system, but there is potential for many more currently unknown microorganisms to be discovered and classified (Schoenian, *et al.*, 2011).

The *Leucoagaricus gongylophorus* Fungi

The *Leucoagaricus gongylophorus* fungi have coevolved over thousands of years with their Attini symbionts. The fungal cultivars are propagated as mycelium, asexually, and are carried to a new colony by the foundress queen. With only one exception, all fungi farmed by the Attini are from the tribe Lepiotaceae (Basidiomycotina: Agaricales: Lepiotaceae), which are predominantly found in tropical environments (Mueller, 1998). This mutualism was procured from 50 million years of coevolution between the two kingdoms to breed these species purely to support each other (Currie, *et al.*, 2003).

Within the fungal gardens, the ants maintain a "vertical gradient of biomass degradation" by distributing fresh leaf litter and debris to the top layer, growing fungi with gongylidia in the middle layer, and storing exhausted substrate in the bottom layer (Huang, *et al.*, 2014). The exhausted substrate removed from the bottom layer is incorporated back into the leaf litter in the top layer (Huang, *et al.*, 2014). *L. gongylophorus* utilize various types of lignocellulases to degrade plant biomass. In addition, lignocellulases degrade other substrate depending on the different products used by the ant species (Aylward, *et al.*, 2013). Hydrolytic enzymes within the gardens are capable of degrading casein, gelatin, starch, cellobiose, and cellulose. These enzymes are not detected in the hyphae of the fungi, so they may be produced by other symbionts in the gardens. The fungi are capable of producing amylases to degrade starch within the gardens (Erthral Jr., *et al.*, 2009). The degradation of these materials allows the fungi to grow and produce gongylidia for the ants.

The production of gongylidia makes for a perfect mutualism between the ants and fungi within the system. The gongylidia provide the larvae and queen with the nutrients they need as their sole food source. Although gongylidia complete the relationship between the two

organisms, it is unsure when they appeared within the phylogeny and how this has affected the coevolution of the leaf-cutter system. It is speculated that the origin of gongylidia was introduced through a persistent viral symbiont vertically transmitted through *L. gongylophorus* ancestors.

The *Totiviridae* Family

Members of the *Totiviridae* family are a type of mycovirus, a virus that infects fungi. There are several viral families that are considered mycoviruses including, but not limited to, *Adenoviridae, Barnaviridae*, *Chrysoviridae, Hypoviridae, Partitiviridae*, *Pseudoviridae*, *Reoviridae*, and *Totiviridae*. Most mycoviruses are icosahedral and are either double stranded RNA or linear positive sense single stranded RNA (Göker, *et. al*, 2011). These viruses are considered persistent within their hosts because they are vertically transmitted, infect their hosts for many generations, and are asymptomatic (Roossinck, 2015). Viruses that are not persistent are considered acute. Acute viruses are transmitted horizontally and cause disease. They can become chronic in the host, but can also be cleared from the host, or result in death of the host (Roossinck, 2012). In contrast, many cases of persistent viruses and their hosts evolve mutualistic relationships over time (Roossinck, 2011).

Members of the *Totiviridae* family are similar to other mycoviruses in that they have icosahedral virions that encapsidate dsRNA. Their genomes typically contain two overlapping open reading frames (ORFs) that encode an RNA dependent RNA polymerase (RdRp) and a major capsid protein (MCP) (Dantas, *et al.*, 2016). Typically, genomes from the *Totiviridae* family are uncapped, linear strands that are 4.6-7.0 kbp in size (Wickner, *et al.*, n.d.) There are five genera within the *Totiviridae* family: *Giardiavirus*, *Leishmaniavirus*, *Trichomonavirus*,

Totivirus, and *Victorivirus*. Only members of the *Totivirus* and *Victorivirus* genera exclusively infect fungi (Dantas, *et al.*, 2016).

Viral Symbionts as Mutualists

It is a common misconception that all viruses are detrimental to their host's fitness. This may be because the first viruses discovered, Tobacco mosaic virus and Yellow fever virus, are extremely harmful pathogens, and because many viruses in the news today are found to reduce host fitness. The majority of viruses are asymptomatic or beneficial in certain circumstances (Pradeu, 2016). These viruses, when persistent, often introduce genetic material to the host that can become helpful for future evolution. If a host is put under abiotic stress, the genetic material introduced by the virus can aid in the host adapting to its change in environment (Roossinck, 2005). As Thomas Pradeu explains in his article on mutualistic viruses, "Mutualism is not a yes/no question, but rather a question of degree, and moreover it is often a contextual question."

Mutualistic viruses can be found throughout the tree of life in mammals, fish, insects, plants, fungi, and bacteria (Roossinck, 2015). Some of the ways that viruses can increase a host's fitness are through development, protection against a pathogen or disease, or invasion of new hosts or niches (Pradeu, 2016). One example of protection against disease is in humans coinfected with hepatitis G virus and HIV. Hepatitis G virus does not show any clinical symptoms and HIV virus is observed to progress slower (Roossinck, 2015, Move Over). An example of invasion of a new niche is some bacteria use lysogenic bacterial phages to eliminate other bacterial competitors when invading new environments (Bossi, *et al.*, 2003).

Both cases of protection against a pathogen or disease and invasion of a new host or niche involve putting the host organism under stress in some way. Before the host is put under these circumstances, the virus may appear to be asymptomatic. Once the ideal circumstances present themselves, the virus is able to aid its host through its supplementary gene products. An example of this is in the mutualistic symbiotic plant-fungi-virus model of Curvularia thermal tolerance virus. Curvularia thermal tolerance virus infects the *Curvularia protuberata* endophytic fungi. With the help of both fungus and virus, plants are able to flourish in geothermal soils reaching average temperatures of 50°C (Roossinck, 2012). In fungi, a type of beneficial totivirus called a killer virus aids its fungal yeast and smut hosts in killing invading strains (Ghabrial, 1998). Both of these cases involve fungi being placed in environments in which they would not survive without their viral counterparts. As the catalogue of asymptomatic mycoviruses expands, it is important to question what circumstances would allow for these viruses to express their hidden genes, and how many fungi contain viruses that are not yet known.

A totivirus was isolated in one strain of *L. gongylophorus* in a lab in Texas (unpublished data). It is hypothesized that the totivirus that aids in the fungi's mutualism with leaf-cutter ants by affecting the production of gongylidia. To test this hypothesis, dsRNA was extracted from *L. gongylophorus* samples from leaf-cutter ant gardens containing gongylidia. This dsRNA was then processed through random priming cloning and reverse transcription PCR. For further analysis, the sample must be cloned and sequenced.

Materials and Methods

Double Stranded RNA Purification

Fourteen samples were collected from fungal gardens and liquid cultivars from the Currie Lab at the University of Wisconsin Madison. These samples were grown in gardens maintained by ants of the *Acromyrmex*, *Atta*, *Myrmicocrypta*, and *Trachymyrmex* genera or were from liquid cultivars of samples from the gardens. The samples were collected after the fungi had grown gongylidia and the gongylidia had proliferated. After the samples were collected, they were lyophilized and stored in -20°C freezer until nucleic acid extraction was performed. Listed in Table 1 is each sample and its detailed information.

Table 1: Attine Sample Descriptions

Sample Number	Colony Name	Attine Species	Weight Before Lyophilization (g)	Weight After Lyophilization (g)
1	Luna	*Acromyrmex echinator*	0.239	0.071
2	Luna	*Acromyrmex echinator*	0.333	0.107
3	Luna	*Acromyrmex echinator*	0.326	0.128
4	Zoe	*Atta cephalotes*	0.310	0.109
5	Zoe	*Atta cephalotes*	0.280	0.111
6	Zoe	*Atta cephalotes*	0.280	0.090
7	Zoe	*Atta cephalotes*	0.957	0.051
8	LK170123-01	*Myrmicocrypta sp.*	1.178	0.022
9	LK170123-01	*Myrmicocrypta sp.*	1.405	0.054
10	LK170123-01	*Myrmicocrypta sp.*	1.327	0.005
11	Luna	*Acromyrmex echinator*	0.604	0.063
12	WM170124-07	*Trachymyrmex sp.*	0.757	0.080
13	AR1701223-03	*Trachymyrmex sp.*	1.522	0.063
14	Zoe	*Atta cephalotes sp.*	N/A	0.003

Extraction was performed through a modified version of Dodds (Dodds, *et al.*, 1984). *Curvularia protuberata* was used as a positive control. Before nucleic acid extraction, 20 mg of each lyophilized sample was ground up using liquid nitrogen. Extraction buffer with freshly added β-mercaptoethanol (900 µL; 5M NaCl, 1M Tris, 0.5M EDTA, 10% SDS, 1 µL β-mercaptoethanol) was added to each sample and mixed thoroughly. Phenol chloroform (900 µL) was added to each sample. Each individual sample was mixed in the vortexer until a milky consistency was reached. The samples were shaken for 10 minutes on the vortexer at high speed and then placed in a centrifuge for 10 minutes at 13,000 rpm.

After centrifugation, the aqueous layer was removed and added to a new 1.5 mL tube and mixed with absolute ethanol (180 µl). Fine cellulose (Cellulose Medium Fibers C6288 Sigma Aldrich) was added to each sample and the tubes were mixed on the vortexer until the cellulose was fully suspended. When consistency was reached, each sample was centrifuged at 5,000 rpm for 2.5 minutes. The aqueous layer was discarded after centrifugation as it contained the total nucleic acids. The dsRNA was bound to the cellulose.

Application Buffer (5M NaCl, 1M Tris, 0.5M EDTA, 16.5% ethanol) was added to each sample and mixed thoroughly. The samples were centrifuged at 5,000 rpm for 2.5 minutes and the aqueous layer was drained. This wash was repeated three more times, draining the aqueous layer each time, to remove all non-bound nucleic acids. After the fourth wash, the tubes were drained for one minute. After draining, 500 µL of elution buffer (5M NaCl, 1M Tris, 0.5M EDTA) was added to each tube and let sit for 10 minutes. After 10 minutes, the mixture was placed in the centrifuge at 5,000 rpm for four minutes.

The remaining aqueous layer was removed, transferred to a fresh tube, mixed with 60 µL of Sodium Acetate (3M) and 80 µl of cold absolute ethanol, and stored overnight at -20°C. After

overnight storage, the sample is spun in a cold centrifuge at 13000 rpm for 10 minutes. A pellet can be observed at the bottom of the tube. The aqueous solution is disposed of, being careful not to disturb the pellet. The pellet is then washed with 70% cold ethanol, vortexed, and centrifuged at 7000 rpm for 5 minutes. This step is repeated with 100% cold ethanol. The pellet is then dried until all traces of ethanol have evaporated. To prepare for gel electrophoresis, the pellet is resuspended in EDTA (0.1 mM, 50 µL). A 1.2% gel electrophoresis analysis of the samples was run using the PSU 100 bp ladder. The PSU 100 bp ladder can be viewed in Appendix A in Figure 3.

Random Priming Cloning and Reverse Transcription Polymerase Chain Reaction

Random priming cloning and Reverse Transcription Polymerase Chain Reaction were performed through a modified version of Marquez (Marquez, *et al.* 2007). Sample 12 (2.5 µl) was mixed with the RT Mixture (Pre-Boil) found in Table 2. This mixture was boiled for 2 minutes, and then immediately placed on ice for 2 minutes. After the mixture was chilled the RT Mixture found in Table 2 was added to each sample. The mixture was incubated for 15 minutes at room temperature and then placed on a 50°C heat block for 1.5 hours. After removing the tubes from the heat block, boiled RNase A (1 µl) was added to each sample and incubated for 15 minutes. The tubes were then placed on an 85°C heat block for 2 minutes. After the tubes were removed from the heat block they were treated with the OMEGA bio-tek E.Z.N.A.© Cycle Pure kit. Once treatment was complete, the samples were stored in -20°C overnight.

Table 2: RT and RT-PCR Mixtures

RT Mixture Pre-Boil		RT Mixture		RT-PCR Mixture	
TE 10 mM	1 µl	10 X mMULV buffer	2 µl	10 X IT buffer	2.5 µl
Dodecaprimer Linker 50 µM	1 µl	mMULV enzyme	1 µl	dNTPs 10 mM	0.5 µl
Water	7.5 µl	dNTPs 10 mM	1 µl	RT Linker Primer	5 µl
[redacted]	[redacted]	Water	4 µl	Taq DNA polymerase diluted	2.5 µl
[redacted]	[redacted]	[redacted]	[redacted]	Water	11.5 µl

After thawing, each sample (3 µl) was mixed with the RT-PCR mixture found in Table 2. Each mixture was transferred to a 10 µl capillary tube through capillary action and sealed at both ends. An Idaho Technology Rapid Cycler was used to run the PCR program. The PCR was run with two programs and two holds. Each program can be found in Table 3. The first hold was set at 72°C for 5 minutes and the second hold was set at 37°C for 5 minutes.

Table 3: RT-PCR Program Settings

	Cycle 1		Cycle 2	
Amount of Cycles	1		40	
Denaturing Temperature and Time	94°C	1 min.	94°C	xx
Annealing Temperature and Time	65°C	xx	45°C	xx
Extending Temperature and Time	72°C	45 sec.	72°C	30 sec.

After the RT-PCR, the samples were run in a 1.2% gel with the PSU 1kb ladder used. The PSU 1kb ladder can be found in Appendix A in Figure 3.

Results

Double Stranded RNA Purification

Five samples of *L. gongylophorus* displayed evidence of dsRNA after dsRNA extraction. These five samples are from liquid cultivars from the Myrmicocrypta and Trachymyrmex gardens. These samples were ran on the same gel because they were of similar nature. Other samples such as samples 1 through 6 contained other tissue from their gardens that was not of fungal origin. The results of the extraction of samples 8, 9, 10, 12, and 13 can be found in Figure 1.

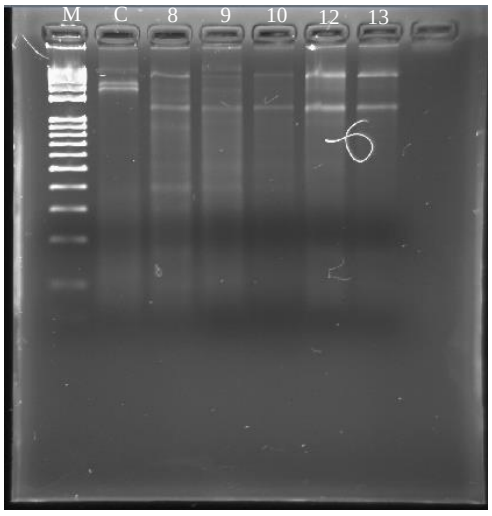

Figure 1: Double Stranded RNA Extraction 1.2% Gel

The sample numbers are displayed above the lanes. M is the PSU 100 bp marker and C is the positive control. In this gel, the samples are in comparison to the 4100 bp mark. This is comparable to average totivirus length between 4700 bp and 6700 bp. As lane 6, the sample from the WM170124-07 garden, is the strongest concentration it was used for RT and RT-PCR. While

the same procedure was performed on the other samples, these five samples were the only samples that presented positive results. In figures 4, 5, and 6 the results of dsRNA extractions in samples from other colonies are displayed. These extractions require further procedures to identify dsRNA and the figures are can be found in Appendix B.

Random Priming Cloning and Reverse Transcription Polymerase Chain Reaction

Sample 12 was used for random priming cloning and RT-PCR. Through the RT, cDNA was synthesized from the dsRNA extracted from the gongylidia. This cDNA was used in RT-PCR. The results of the RT-PCR are displayed in Figure 2. Lane 5 is sample 12 compared to the PSU 1 kb ladder in lane 1.

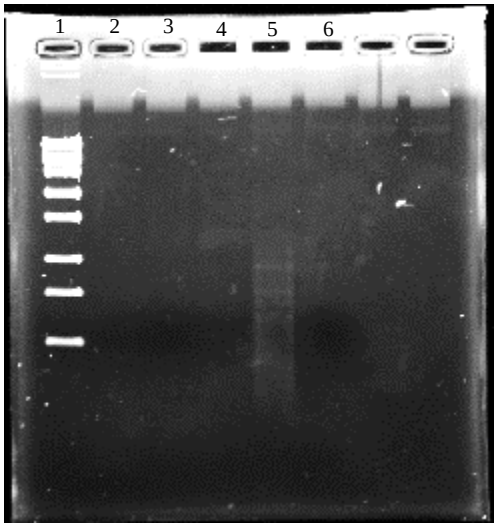

Figure 2: RT-PCR Results on 1.2% Gel

The PCR product from Sample 12 can be used for further cloning and sequencing due to the smear observed in Figure 2.

Discussion

The products found within the gel after dsRNA extraction are at the length typically expected of *Totiviridae*, between 4.6-7.0 kbp in length. Each sample provided the same pattern of bands, so it is concluded that the dsRNA extracted from each sample is of similar derivation. Further processing was needed to confirm the origin of the nucleic acid identified, so sample 12 was processed through random priming cloning to prepare for RT-PCR. Sample 12 was chosen because it had the highest apparent concentration. With such small samples of fungi, results with high concentrations are imperative.

The results of the RT-PCR can be observed in the gel in Figure 2. A smear can be observed in lane 5 in low concentration. This PCR product will be used for further processing to determine if it is a totivirus. The cDNA created through RT-PCR cannot immediately be sent for sequencing. The cDNA must be cloned and have many copies to be sequenced. In order to create a sufficient number of clones, the cDNA is inserted into a vector through ligation. After ligation, transformation is performed to insert the vector into a bacterial cell for cloning. Bacterial cells have a notorious ability to generate many clones of DNA at once through rapid cell proliferation (Carter, 2015). Bacterial colonies transformed with a ligated vector undergo a miniprep to extract the plasmid DNA, and the DNA is used for sequenced analysis. The procedure to conduct the cloning and sequencing of the dsRNA extracted from the *L. gongylophorus* samples following RT-PCR is detailed below.

The vector used for the *L. gongylophorus* samples is a vector supplied by Promega Corporation. It is the pGEM®-T Easy vector which is ideal for high-copy-number vectors and has 3'-terminal thymidine at both ends to improve ligation efficiency. In addition, the vector allows for easy screening of which bacterial colonies contain the clones through blue/white coloration (Promega, 2015). The cDNA is mixed into a ligation reaction solution that utilizes DNA ligase to insert the cDNA into the vector (Carter, 2015). The ligation reaction solution that is taken from the Promega Technical Manual: pGEM®-T and pGEM®-T Easy Vector Systems can be observed in Table 4 in Appendix A. After mixing with the ligation reaction solution, the solution is incubated overnight at 4°C in order to achieve the maximum number of transformants (Promega, 2015).

Before performing transformation, four plates are prepared. Two plates will be LB/ampicillin/IPTG/X-Gal for the ligation reaction and two plates will be used to observe transformation efficiency (Promega, 2015). LB plates are made up of Luria Broth agar. X-Gal and IPTG are two solutions that are used for blue-white plate screening (Padmanabhan, 2011). Bacterial colonies that contain the successful transformation of the plasmid will be blue, while all other colonies will be white. This occurs because the colonies that contain the plasmid also contain the lacZ operon. This operon produces β-galactosidase to metabolize lactose. The X-Gal that is spread on the plates will be hydrolyzed in the presence of β-galactosidase and then dimerize to produce a blue pigment (Padmanabhan, 2011). The colonies without the plasmid will not contain the lacZ operon and will not turn blue in the presence of X-Gal.

Ampicillin is spread across the plates, because the bacteria used for transformation will be resistant to it if they have acquired the plasmid. This is to ensure that only cells that have acquired the plasmid will grow on the plates (Carter, 2015). The bacteria cells used in this

procedure are *Escherichia coli* cells sensitive to ampicillin (Promega, 2013). These cells are extremely fragile and must be handled gently throughout the transformation process. Once the transformation mixture is created with the cells and the ligation reaction, it must be kept on ice, heated in a water bath, and then incubated in SOC medium at 37°C for 1.5 hours (Promega, 2015). Incubating in SOC medium allows a maximum number of transformations (Hanahan, 1983). Plates are incubated overnight for 16-24 hours at 37°C and result in approximately 100 blue colonies (Promega, 2015).

The blue colonies of interest will be identified from the white colonies on the plates (Promega, 2015). These colonies contain copies of the recombinant DNA and must be purified from the *E. coli* cells for sequencing. This is done through a miniprep that lyses the bacteria, extracts the plasmid DNA, and purifies it (Promega, 2015). After the miniprep, the purified DNA can be sent for sequence analysis. After sequencing, the nucleotide data can be processed through BLAST and Geneious analysis. From this data, the size of the genome, number of ORFs, and location of the RdRp and MCP within the nucleic acid can be identified. This information will allow conclusions to be made on the type of virus, specifying if it is a totivirus. Further analysis through phylogeny would provide insight into the virus' origin and closest relatives.

The element of a commensal or mutualistic totivirus within this multipartite symbiotic system would be revolutionary. In the leaf-cutter ant symbiosis, there are three main organisms in the system, but additional microorganisms allow for the system to be maintained. The role of this potential virus is not yet known, as there is much more to be discovered within its genome, but it is possible that the virus could have introduced genes into *L. gongylophorus* that allowed the symbiosis to flourish from the start. It has been suggested that the virus could aid in

production of gongylidia, which would be crucial in maintaining the symbiotic system. As the system is studied further the extent of this symbiotic relationship will become more apparent.

Appendix A

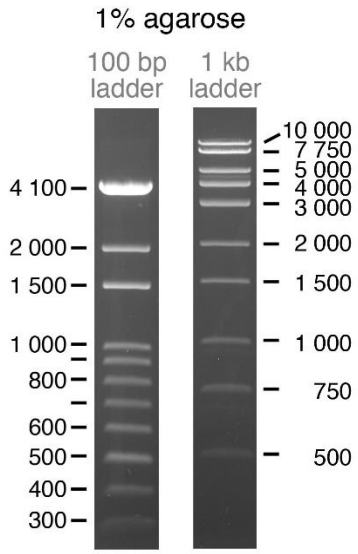

Figure 3. Penn State Nucleic Acid Ladders

This diagram and the PSU Nucleic Acid Ladders used in this study are taken from Henrici (Henrici, *et al.*, 2017).

Table 4: Ligation Reaction Solutions Supplied by Promega Technical Manual: pGEM®-T and pGEM®-T Easy Vector Systems

Reaction Component	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50ng)	1 µl	1 µl	1 µl
PCR Product	3 µl	xx	xx
Control Insert DNA	xx	2 µl	xx
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
Water	0 µl	1 µl	3 µl
Total Volume	10 µl	10 µl	10 µl

Appendix B

Figures 4, 5, and 6 display the extractions of samples 1, 2, and 7. These figures were considered to be unsuccessful for various reasons. Samples 1, 2, and 3 were from the Luna colony and contained other tissue besides the fungal tissue such as what appeared to be plant tissue and feces. The process used for extraction had been optimized for fungal tissue, so some of the other tissue may have not been removed from the sample. Samples 4, 5, 6, and 7 were from the Zoe colony. Samples 4, 5, and 6 were similar to samples 1, 2, and 3 in that there were other tissues within the samples. After the failed extractions of samples 1 and 2, extractions for samples 3 through 6 were not conducted. Sample 7 had been grown in liquid media and lyophilized and did not contain any other tissue. Extraction was performed on this sample for this reason.

In Figure 4, sample 1 is in lane 3. As the sample is measured to be a larger nucleic acid, it was concluded not to be dsRNA, and most likely DNA from the foreign tissue that had not been removed during the extraction. With further processing within the phenol chloroform step, this issue may have been resolved. In Figure 5, sample 2 is in lanes 3 and 4. A similar band is observed and it is likely due to the same reason. In Figure 6, sample 7 was inserted in lane 3. Due to contamination, the dsRNA was concluded to be destroyed by RNase contamination.

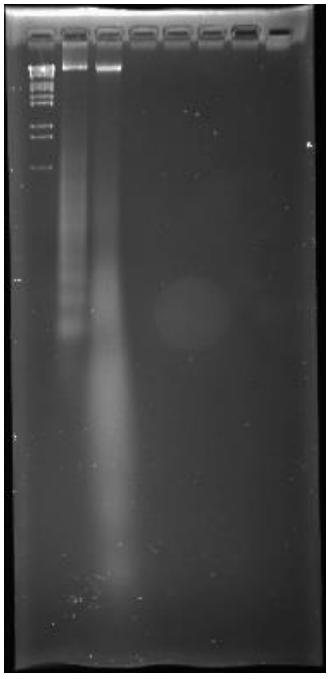

Figure 4: Double Stranded RNA Extraction of Sample 1 in 1.2% Gel

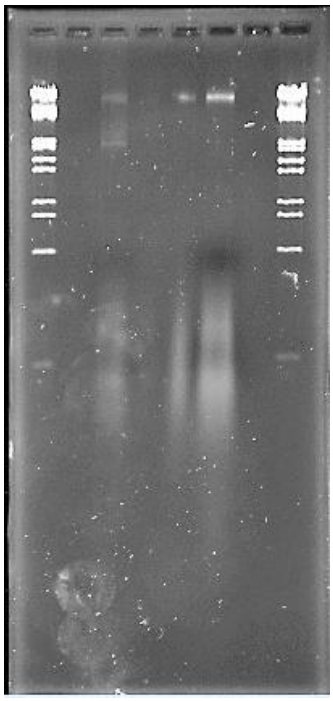

Figure 5: Double Stranded RNA Extraction of Sample 2 in 1.2% Gel

Figure 6: Double Stranded RNA Extraction of Sample 7 in 1.2% Gel

BIBLIOGRAPHY

Aylward, F. O., Burnum-Johnson, K. E., Tringe, S. G., Teiling C., Tremmel, D. M., Moeller, J. A., Currie, C. R. (2013, 04). Leucoagaricus gongylophorus Produces Diverse Enzymes for the Degradation of Recalcitrant Plant Polymers in Leaf-Cutter Ant Fungus Gardens. *Applied and Environmental Microbiology, 79*(12), 3770-3778. doi: 10.1128/aem.03833-12

Bossi, L., Fuentes, J. A., Mora, G., & Figueroa-Bossi, N. (2003, 10). Prophage Contribution to Bacterial Population Dynamics. *Journal of Bacteriology, 185*(21), 6467-6471. doi:10.1128/jb.185.21.6467-6471.2003

Carter, Matt (2015). "Molecular Cloning and Recombinant DNA Technology" in *Guide to Research Techniques in Neuroscience (0-12-800511-4, 978-0-12-800511-8),* (p. 219)

Currie, C. R. (2003, 01). Ancient Tripartite Coevolution in the Attine Ant-Microbe Symbiosis. *Science, 299*(5605), 386-388. doi:10.1126/science.1078155

Currie, C. R., Mueller, U. G., & Malloch, D. (1999, 07). The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences, 96*(14), 7998-8002. doi:10.1073/pnas.96.14.7998

Currie, C. R. (2001, 10). A Community of Ants, Fungi, and Bacteria: A Multilateral Approach to Studying Symbiosis. *Annual Review of Microbiology, 55*(1), 357-380. doi:10.1146/annurev.micro.55.1.357

Currie, C. R., Scott, J. A., Summerbell, R. C., & Malloch, D. (1999, 04). Erratum: Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature, 398*(6729), 701-704. doi:10.1038/19519

Dantas, M. D., Cavalcante, G. H., Oliveira, R. A., & Lanza, D. C. (2016, 01). New insights about ORF1 coding regions support the proposition of a new genus comprising arthropod viruses in the family Totiviridae. *Virus Research, 211*, 159-164. doi:10.1016/j.virusres.2015.10.020

Dimijian, Gregory G. "Evolving Together: The Biology of Symbiosis, Part 1." *Baylor University Medical Center Proceedings* 13, no. 3 (07 2000): 217-26. doi:10.1080/08998280.2000.11927677.

Dodds, J. A., Morris, T. J., Jordan, R. L. (1984, 01). Plant Viral Double-Stranded RNA. *Annual Review of Phytopathology, 22*(1), 151-168. doi:10.1146/annurev.phyto.22.1.151

Erthal, M., Silva, C. P., Cooper, R. M., & Samuels, R. I. (2009, 01). Hydrolytic enzymes of leaf-cutting ant fungi. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 152*(1), 54-59. doi:10.1016/j.cbpb.2008.09.086

Ghabrial, S. A. (1998). *Virus Genes,16*(1), 119-131. doi:10.1023/a:1007966229595

Göker, M., Scheuner, C., Klenk, H., Stielow, J. B., & Menzel, W. (2011, 07). Codivergence of Mycoviruses with Their Hosts. *PLoS ONE, 6*(7). doi:10.1371/journal.pone.0022252

Hanahan, D. (1983, 06). Studies on transformation of Escherichia coli with plasmids. *Journal of Molecular Biology, 166*(4), 557-580. doi:10.1016/s0022-2836(83)80284-8

Henrici, R. C., Pecen, T. J., Johnston, J. L., & Tan, S. (2017, 05). The pPSU Plasmids for Generating DNA Molecular Weight Markers. *Scientific Reports, 7*(1). doi:10.1038/s41598-017-02693-1

Huang, E. L., Aylward, F. O., Kim, Y., Webb-Robertson, B. M., Nicora, C. D., Hu, Z., Burnum-Johnson, K. E. (2014, 04). The fungus gardens of leaf-cutter ants undergo a distinct

physiological transition during biomass degradation. *Environmental Microbiology Reports, 6*(4), 389-395. doi:10.1111/1758-2229.12163

Marquez, L. M., Redman, R. S., Rodriguez, R. J., & Roossinck, M. J. (2007, 01). A Virus in a Fungus in a Plant: Three-Way Symbiosis Required for Thermal Tolerance. *Science, 315*(5811), 513-515. doi:10.1126/science.1136237

Miyashira, C., Tanigushi, D., Gugliotta, A., & Santos, D. (2010, 06). Comparison of radial growth rate of the mutualistic fungus of Atta sexdens rubropilosa forel in two culture media. *Brazilian Journal of Microbiology, 41*(2), 506-511. doi:10.1590/s1517-83822010000200035

Mueller, U. G. (1998, 09). The Evolution of Agriculture in Ants. *Science, 281*(5385), 2034-2038. doi:10.1126/science.281.5385.2034

Padmanabhan, S., Banerjee, S., Mandi, N. (2011). Screening of Bacterial Recombinants: Strategies and Preventing False Positives. In G. Brown (Ed.) *Molecular Cloning – Selected Applications in Medicine and Biology* (3-20). India: Intech.

Pradeu, T. (2016, 10). Mutualistic viruses and the heteronomy of life. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences, 59*, 80-88. doi:10.1016/j.shpsc.2016.02.007

Promega. (2013). E. coli Competent Cells: Quick Protocols. Madison, WI: na.

Promega. (2015). Technical Manual: pGEM®-T and pGEM®-T Easy Vector Systems. Madison, WI: na.

Reynolds, H. T., & Currie, C. R. (2004, 09). Pathogenicity of Escovopsis weberi: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia, 96*(5), 955-959. doi:10.1080/15572536.2005.11832895

Roossinck, M. J. (2015, 01). Metagenomics of plant and fungal viruses reveals an abundance of persistent lifestyles. *Frontiers in Microbiology, 5*. doi:10.3389/fmicb.2014.00767

Roossinck, M. J. (2015, 04). Move Over, Bacteria! Viruses Make Their Mark as Mutualistic Microbial Symbionts. *Journal of Virology,89*(13), 6532-6535. doi:10.1128/jvi.02974-14

Roossinck M.H. (2012) Persistent Plant Viruses: Molecular Hitchhikers or Epigenetic Elements?. In: Witzany, G. (eds) *Viruses: Essential agents of life*. Springer, Dordrecht

Roossinck, M. J. (2005, 10). Symbiosis versus competition in plant virus evolution. *Nature Reviews Microbiology, 3*(12), 917-924. doi:10.1038/nrmicro1285

Roossinck, M. J. (2011, 01). The good viruses: Viral mutualistic symbioses. *Nature Reviews Microbiology, 9*(2), 99-108. doi:10.1038/nrmicro2491

Schoenian, I., Spiteller, M., Ghaste, M., Wirth, R., Herz, H., & Spiteller, D. (2011, 01). Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proceedings of the National Academy of Sciences, 108*(5), 1955-1960. doi:10.1073/pnas.1008441108

Totiviridae. (n.d.). Retrieved from https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsrna-viruses-2011/w/dsrna_viruses/191/totiviridae

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