Interaction between the fungus-growing ant *Cyphomyrmex minutus* and its symbionts at Cambalache forest, Puerto Rico

by

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ABSTRACT

The ants in the tribe Attini cultivate a fungus (Basidiomycota: Agaricales) as food and protect it from specific mycoparasites, *Escovopsis* (Ascomycota: Hypocreales), using the antibiotic production capacity of Actinobacteria (*Pseudonocardia*) associated with its exoskeleton. Attini nests are not axenic environments; several other microorganisms (bacteria and fungi) with undescribed roles interact with the ant and the principal symbionts. In addition, the ants show characteristic hygienic behaviors that include farming and grooming of the cultivar and creating, rearranging, and transporting piles of organic refused material in and out of the nest. Currently, 5 different agricultural practices have been described among the Attini and only the members of the *Cyphomyrmex rimosus* group maintain their cultivar in yeast form. All other groups of the Attini cultivate their fungi in mycelial form. Although the interaction in the attine ant symbiosis has been extensively studied, the yeast-cultivating ants and their microbial associates have not been described. In Puerto Rico, *Cyphomyrmex minutus* is the only attine species that practices yeast agriculture. We investigated the microbial community associated with *C. minutus* including the specific cultivar, the possible mycoparasite and the Actinobacteria. We sampled a total of 26 nests of *C. minutus* during the Dry and Rainy seasons at Cambalache Tropical Forest in Puerto Rico. A combination of culture-dependent and independent techniques was used to describe the fungi and Actinobacteria isolated from different components of the nest. We identified the yeast cultivar by sequencing the 28S rDNA gene. We also isolated and identified the fungi associated with the cultivar using morphology and ribosomal operon ITS sequencing. Furthermore, we created a clone library of the fungal ITS region from the organic refuse material in search of pathogens. Actinobacteria genera from the ant exoskeleton and the cultivar were analyzed using 16S rDNA gene. The microbial community associated with *C. minutus* differs significantly from other attine ants. The specific pathogen, *Escovopsis*, was not found in association with the cultivar nor the refuse material. *Pseudonocardia* was not the prevalent actinobacterium genus in the association, but instead *Streptomyces* strains were commonly recovered. Our studies strongly support the hypothesis that the ant maintains the cultivar in yeast form as an adaptation to escape pathogen infection.

Interacción entre la hormiga cultivadora de hongos Cyphomyrmex minutus y sus simbiontes en el bosque de Cambalache, Puerto Rico

RESUMEN

Las hormigas en la tribu Attini cultivan un hongo (Basidiomycota: Agaricales) como fuente de alimento y lo protegen del micoparásito específico *Escovopsis* (Ascomycota: Hypocreales) utilizando la capacidad de producción de antibióticos de actinobacterias asociadas a su exoesqueleto (*Pseudonocardia*). Los nidos de las Attini no son ambientes axénicos en adición a los principales simbiontes varios microorganismos (bacterias y hongos), han sido reportados interactuando con los diferentes componentes del nido y sus simbiontes. Los roles de estos microorganismos permanecen sin describir. Las Attini presentan comportamientos higiénicos característicos que incluyen la inspección y el aseo del cultivar y la creación, rearreglo y transportación de pilas de material orgánico considerado como desecho fuera del nido. Hasta el momento se han identificado 5 tipos de agricultura entre las Attini; solamente las especies del grupo *Cyphomyrmex rimosus* mantienen su cultivar a manera de levadura, los otros 4 grupos cultivan su hongo como micelio. A pesar de que la interacción entre las hormigas Attini y sus simbiontes ha sido extensamente estudiada, las hormigas que cultivan a manera de levadura y los microorganismos asociados a éstas permanecen sin describir. En Puerto Rico, *Cyphomyrmex minutus* es la única especie de Attini que practica agricultura de levaduras. En este estudio se describió la comunidad microbiana asociada a *C. minutus* incluyendo el cultivar, el micoparásito y las actinobacterias. Un total de 26 nidos de *C. minutus* fueron muestreados durante las épocas seca y lluviosa en el bosque de Cambalache en Puerto Rico. Una combinación de técnicas independientes y dependientes de cultivo fueron utilizadas para describir la comunidad de hongos y actinobacterias asociadas a diferentes componentes del nido. El cultivar fue identificado mediante la secuenciación del gen 28S rADN. Además se aislaron e identificaron los hongos asociados al cultivar utilizando caracteres morfológicos y secuenciación de la región ITS del operon ribosomal. Se creó una biblioteca de clones (región ITS del rDNA) del material de desecho que permitió describir la comunidad de hongos asociados a dicho sustrato en busqueda de patogenos. Por otro lado para la identificación de las actinobacterias asociadas al exoesqueleto y al cultivar de *C. minutus* se analizó el gen 16S rADN. La comunidad microbiana asociada a *C. minutus* difiere significativamente de las descritas para otras Attini. El micoparásito, *Escovopsis*, no fue detectado en asociación al cultivar o al material de desechos de *C. minutus*. *Pseudonocardia* no fue el género de Actinobacteria prevalente en asociación con esta Attini. Por el contrario, cepas de *Streptomyces* fueron comúnmente recuperadas. Nuestros estudios apoyan la hipótesis de que las Attini mantienen el cultivar a manera de levadura como una adaptación para prevenir infección de patógenos.

DEDICATION

To those who believed in me during the whole process…

To the ones that love and supported me in every step of the way…

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1. INTRODUCTION: FUNGUS-GROWING ANTS SYMBIOSIS MODEL

1.1. Attini ants and their cultivar

Fungus-growing ants have been described as an example of complex symbiotic interactions with a long history of coevolution. All fungus-growing ants belong to the tribe Attini (Hymenoptera: Formicidae: Myrmicinae). The tribe Attini is estimated to have over 230 species divided in 12 different genera that are in an obligate symbiotic relationship with basidiomycetous fungi (Currie 2001a, Brady and Shultz 2008, Mehdiabadi and Schultz 2010). This association started about 50 milion years ago in the Neotropical region of America during the Eocen (Weber 1958, Brady and Shultz 2008). The cultivar (Agaricales: Lepiotaceae) is the main souce of food for the whole nest. The cultivar is vertically transmitted by the queen to the new nest in its infrabuccal pockets. This practice creates a clone cultivar making it more vulnerable to specific pathogens (Currie 2001b). In some of the basal Attini groups, the cultivar was acquired horizontally in at least two different occasions after their obligatory interaction began (Gerardo et al. 2004, Gerardo et al. 2006). The cultivar has been recently isolated as free-living mycelia fungi (Vo et al. 2009) indicating the interaction is not obligate for this fungi. The phylogenetic relationship between the ant and the cultivar demonstrates that there is a complex dynamic of coevolution not necessarily one to one for all the species (Mikheyev et al. 2006, Vo et al. 2009, Mikheyev et al. 2010,).

The Attini ants engage a significant effort to maintain the cultivar healthy. Each ant genus presents different behavioral traits in relation to the type of agriculture and nest arrangement they performed. Most nests are in soil, but some species use leaf litter, rocks and wood to create storage cavities (Weber 1958, Currie 2001a). The ants maintain the queen safe and

apart from the rest of the colony, the broods, the cultivar and the refuse organic material. Most of the Attini maintain their garden as filamentous fungi that grow using the organic matter that the ants collect from the environment or cut from the plant, depending on the genera (Currie 2001a). Ant social activities revolve around the survival of the colony and maintenance of the cultivar. These activities include collecting organic matter, fertilizing, weeding, rearranging refuse material piles as well as protecting the cultivar from pathogens using antimicrobial chemicals and photolytic enzymes (Weber 1958, Martin 1970, Muller et al. 1998, Currie 2001a).

1.2. Agriculture practice in the Attini

Fungus growing ant species are divided into five major groups (Table 1.1). The tribe Attini was reclassified using a combination of genetic markers and agriculture practice of the ants (Shultz and Brady 2008). The most primitive are the Lower agriculture ants (*Myrmicocrypta*, *Mycocepurus* and some species of *Apterostigma*). Typically, the members of this group cultivate a paraphyletic fungus (*Leucocoprineae*), which could be found as a free-living species. The second group is the Coral fungus agriculture ants (*Apterostigma* species) which are specialized on maintaining a fungus of the *Pterulaceae* family (Coral fungus) different from the other fungus-growing ant's cultivars.

The next group is the Yeast agriculture ants (*Cyphomyrmex rimosus* group) that cultivate small and irregular clusters of yeast (Snelling and Longino 1992). The yeast cultivar in *Cyphomyrmex rimosus* group is a monophyletic *Leucocoprinea* that can grow in a mycelial phase when free-living (Vo et al. 2009). The more evolved Attini ants are the higher agriculture ants (*Trachymyrmex* & *Sericomyrmex*) and the leaf cutters ants (*Atta* &

Acromyrmex). These groups have a *Leucocoprinea* cultivar that cannot live without the ants and produce specialized structures, gogylidia, which the ants consume (Weber 1958).

The only genera of fungus-growing ants recorded for Puerto Rico are *Mycocepurus*, *Mycetophylax* (Lower Attini), *Trachymyrmex* (Higher Attini) and *Cyphomyrmex* (Lower Attini, yeast agriculture) (Weber 1972 , Osorio-Pérez 2007). Of these, *Cyphomyrmex minutus* is the only species reported for Puerto Rico that practices yeast agriculture (Snelling and Longino 1992, Shultz and Brady 2008).

Table 1.1: Summary of agriculture practices by the fungus-growing ants

Attini	Agriculture	Attini representative species	Cultivar	Pathogen
Lower	**Lower**	*Mycocepurus smithi, M. tardus, M.curvisoibosus* *Myrmicocrypta infuscata, Myr. buenzlii,* *Myr. ulrichi and Myr. ednaella* *Cyphomyrmex constatus, C. muelleri and C. longiscapus* *Apterostigma auriculatum*	*Leucocoprineae*	*Escovopsis sp*
	Coral Fungus	*Apterostigma dentigerum, A. dorotheae, A. collare, A. manni*	*Pterulaceae*	*Escovopsis sp*
	Yeast	*Cyphomyrmex minutus, C. rimosus and* *C. cornutus*	*Leucocoprineae*	not found
Higher	**Higher**	*Sericomyrmex parvulus* *Trachymyrmex zeteki , T. papulatus,* *T. opulentus, T. smithi*	*Leucocoprineae*	*Escovopsis sp*
	Leaf-cutter	*Acromyrmex versicolor and Acro. Octospinosus* *Atta cephalotes, Atta laevigata, Atta mexicana and Atta texana*	*Leucocoprineae*	*Escovopsis sp*

*Based on Shultz & Brady 2008; Mehdiabadi and Shultz *2009;* Mikheyev et al. 2010

1.3. The cultivar-specific pathogen *Escovopsis*

Attine nests are far from being sterile environments the clonally spread cultivar is vulnerable to opportunistic pathogens and parasites. The specific parasite of the cultivar is the microfungus *Escovopsis* (Ascomycota: Hypocreales), which is horizontally transmitted from

one generation to the other and cannot be isolated from the environment as a free-living organism (Currie 2001b, Reynolds and Currie 2004, Gerardo et al. 2004). The transmission of the cultivar is an evident exploitation of the ant-cultivar mutualism system (Currie 1999b, Reynolds and Currie 2004). *Escovopsis* is a necrophitic parasite that secretes specific compounds to invade the cultivar mycelium (Reynolds and Currie 2004).
An uncontrollable growth of the pathogen can slow the production rate of new workers and the growth of the cultivar in the ant colony. Without the ant, the pathogen overgrows and devastates the fungal garden in a few weeks (Currie 2001b). The parasite *Escovopsis* is unknown from yeast agriculture (Table 1.1), but Schultz and Brady (2008) suggest that the morphology of the yeast cluster influences the pathogenicity of the parasite.

1.4. The Actinobacteria symbionts

As another adaptation to protect the cultivar, the ants live in association with Actinobacteria; during their evolution, the ants developed the capacity to keep antibiotic-producing bacteria in crypts located in the propleural plates supplemented by products of internal secretion glands (Weber 1966, Currie 2001a, Little et al. 2003, Currie et al. 2006). The Actinobacteria is a big group of Gram-positive filamentous bacteria with special lipids in their membrane, making them resistant to environmental conditions. Actinobacteria were described, over the years, as bacteria with special adaptations: production of secondary metabolites, degradation of complex polysaccharides and resistance to weather changes (Brenner et al. 2005). In addition, the group naturally produces antibiotic substances that can kill other bacteria, fungi and some small protists (Brenner et al. 2005). These characteristics confer important evolutionary advantages as symbionts (Currie et al. 2006). The specific Actinobacteria strain acquired by the ants is vertically transmitted to the next generation with occasional free-living acquisition

that results in strain diversification between ant species (Poulsen et al. 2007, Cafaro et al. 2011). The specificity is important because it ensures the health of the cultivar as the bacteria defend it from the pathogen *Escovopsis*. Free-living bacterial strain acquisition can be considered an advantage that preserves the efficacy of the antibiotic product (Poulsen et al. 2005, Cafaro et al. 2011)**.**

The higher Attini (Table 1.1) genera do not present the crypts as a part of their anatomy, but the Actinobacteria seem to be present in other parts of the exoskeleton. Some species present a visible powdery white coat of Actinobacteria in the exoskeleton. Recent studies have shown that the most frequently isolated Actinobacteria are *Pseudonocardia* species, but other genera, such as *Streptomyces* and *Amycolatopsi*s have also been isolated in high frequency (Cafaro and Currie 2005, Sen et al. 2009, Boosma et al. 2009, Fernández-Marín et al. 2009). The common denominators between those genera are their close phylogenetic relationships, high frequency in fungus-growing ants and their antibiotic production potential (Gerardo et al. 2006, Cafaro and Currie 2005, Cafaro et al. 2011, Mehdiabadi and Shultz 2010).

1.5. An overview of the fungus-growing ant symbiosis basic model

Figure 1.1 shows a graphic representation of the interaction between the fungus-growing ants and their symbionts. The fungus-growing ants cultivate Basidiomycota fungi in a mutualistic relationship. In exchange for food the ants provide the cultivar optimal growth conditions, substrate and constant grooming. Beside multiple defense mechanisms and hygienic behaviors the cultivar can be parasited by *Escovopsis* (this has not been shown for yeast agriculture ants) (Currie 2001b). Other opportunistic microfungi are also present in the nest and can be affected by these defense mechanisms (Fernández-Marín et al. 2009). The cultivar is essential for the colony, and thus, the ants have developed a direct interaction with Actinobacteria that live in the exoskeleton to protect the cultivar. The Actinobacteria gets protection and nutrients from the ant (Currie et al. 2006) and the ants benefit from the Actinobacteria naturally produced antibiotics (Currie 2001a, 2006, Cafaro and Currie 2005, Mueller et al. 2008). As a consequence, the Actinobacteria have an antagonistic relationship with the cultivar parasite *Escovopsis*. Indirectly, the cultivar and the ant are both positively affected by the unidirectional Actinobacteria-*Escovopsis* antagonism (Figure 1.1).

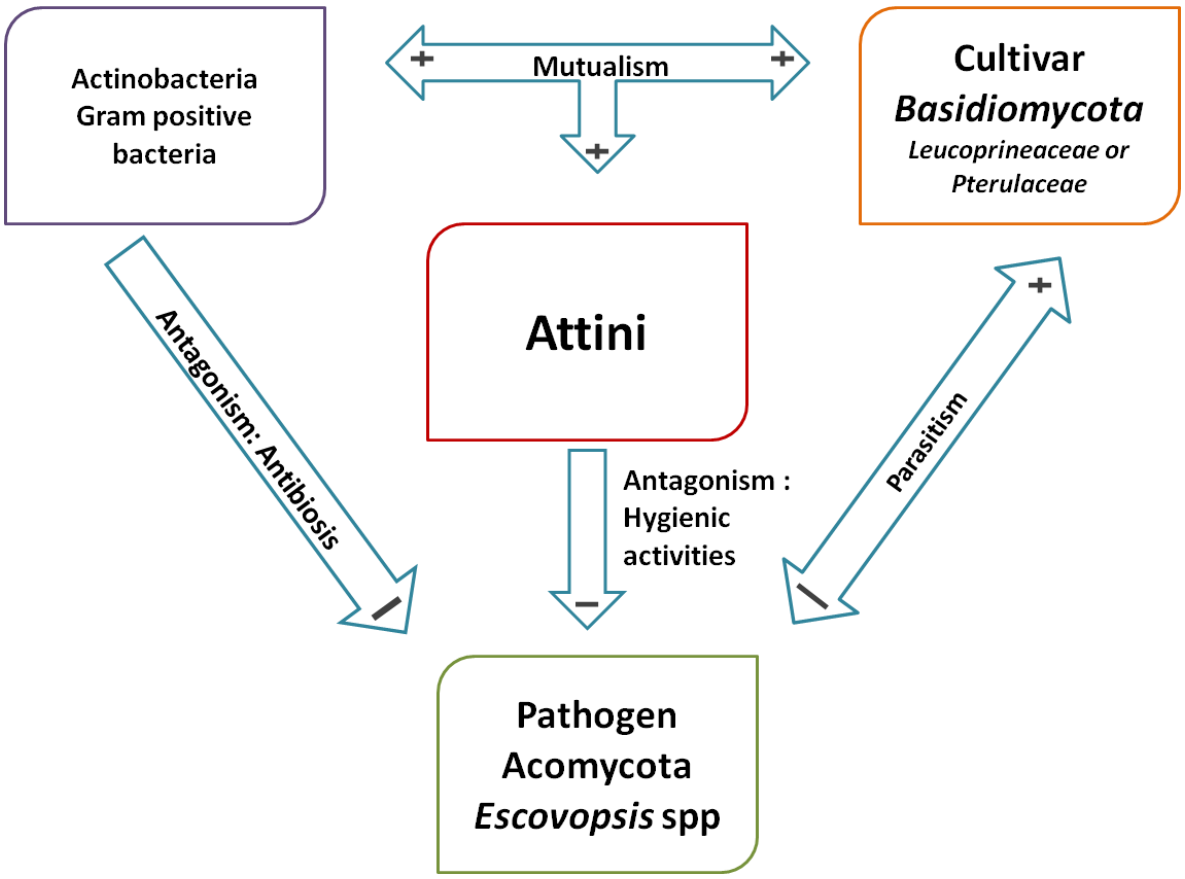

Figure 1.1: Fungus-growing ant basic model base on the information published by Weber 1958, Currie 2001a, Shultz and Brady 2008 and Mehdiabadi and Shultz 2010.

1.6. Yeast agriculture and *Cyphomyrmex minutus*

Cyphomyrmex species are part of an evolutionarily lower intermediate group of fungus-growing ants of 39 identified species (Schultz and Brady 2008, AntWeb 2012). *Cyphomyrmex* ants are the smaller Attini ants that construct simple nests with only one chamber. The ants collect caterpillars, dead insect and feces as substrates for the cultivar (Weber 1958). This genus is divided into three different groups based on their phylogeny: *muelleri*, *stiagatus* and *rimosus* (Schultz and Brady 2008, Mehdiabadi and Shultz 2010). Members of the *muelleri* and *stiagatus* groups practice lower agriculture, which means that the Leucocoprinae fungi that they consume grow as mycelium in the nest.

All members of the *rimosus* group have the capacity to maintain the fungus cultivar in a yeast phase even when it is present as mycelia in the environment (Chapela et al. 1994). They cultivate a separate clade of leucocoprinaceus fungi different from the clade of other Lower Attini (Shultz and Brady 2008). *Cyphomyrmex* species use yeast as their primary source of food and preserve it that way.

Information about the Attini yeast agricultural practice is limited. Most is an inconclusive extrapolation of previous studies made with Higher Attini ants (Table 1.1). Contemporary studies demonstrate that assumptions are not well supported in all cases. Each type of Attini agriculture practice possesses its own specific adaptations. This does not deny important similarities, resulting from millions of years of coevolution. All the Attini maintain a cultivar that is vertically transmitted and protected by weeding, grooming and rearrangement of the nest.

In addition, the ants protect the cultivar using internal and external mechanisms that vary between agricultural practices. Attini ants present a visual white cover of an antibiotic producing Actinobacteria on their exoskeleton. In some cases the ant also has specific fovea structures to bring protection to the bacterial symbiont. It is currently know that Lower agriculture *Cyphomyrmex* species (*C. constatus* and *C. levigatus*) present fovea structures at the propleural plate close by glandular secretion cavities as well as an actinobacteria white coverage (Currie et al. 2006). This adaptation has not been explored for yeast agriculture ants.

Another unexplored adaptation is the yeast agriculture system. We do not know why or how the *rimosus* group maintains the cultivar as yeast. Some authors suggest that the ant obligate the cultivar to grow as yeast by affecting the environmental conditions (Mehdiabadi and Shultz 2010).

The other important question about the yeast agriculture is: Where is the pathogen? There is no evidence of *Escovopsis* in association with the *rimosus* group. Two important points are worth mentioning (1) there are no studies about yeast agriculture pathogens and (2) *Escovopsis* (cultivar pathogen) can only be found in association with the cultivar in 39.7% (average) of the eight sampled genera of other Attini (Currie 2001b). Based on this information, few scenarios can be possible: i) *Escovopsis* cannot affect the cultivar in the yeast phase because of unknown anti-infection mechanisms; ii) *Cyphomyrmex* ants have very efficient and undescribed mechanisms to defend the cultivar; iii) there is another pathogen for this group of Attini *rimosus* group. The first two possibilities have not been explored. The latter scenario can be supported by recent studies about other fungi in association with fungus-growing ants nests (Rodrigues et al. 2005a, Rodrigues et al. 2008, Pagnocca et al. 2008, Rodrigues et al. 2009). Nevertheless, *Escovopsis* is the only genus that passed Koch's postulates for pathogenicity among all isolated fungi (Currie 1999b, Currie 2001b).

Because yeast agriculture symbiosis interactions and defense mechanisms are unexplored further studies are needed to understand this agriculture practice and the microorganisms involved in the system. Also, how does this agriculture practice fit into the fungus-growing ant symbiosis model?

1.7. Project summary and Objectives

The isolation, identification and organization of all the species that are part of the interaction between the yeast agriculture Attini, *C. minutus* and its cultivar represent an important study about diversity, evolution and symbiosis. The principal objective of this study is to

characterize the relationship between the fungus-growing ant *Cyphomyrmex minutus* and its symbionts in Cambalache Tropical Forest, Puerto Rico. The morphological and adaptive characteristics of *C. minutus* and its associated microorganisms make an exceptional interaction and pose many questions about this symbiosis.

We hypothesize that *C. minutus*, like other fungus-growing ants, have multiple evolutionary adaptations, including symbiosis, to protect their agricultural practice.

In Chapter 2 this study concentrated in the description and analysis of *C. minutus* and its yeast cultivar. We start with a description of the environment and then the microorganisms in the association. In this chapter we identify the Attini ant by morphology. We also describe the nest and the ant behavior under natural and laboratory conditions. We identify and describe the yeast cultivar using a similar approach including SEM and light microscopy.

In Chapter 3, we describe morphological characteristics of *C. minutus* exoskeleton that permit the presence of Actinobacteria associates. We present our results about the presence/absence of Actinobacteria in the exoskeleton, specifically the propleural plates of the ant. This chapter includes a description of the Actinobacteria diversity in a phylogenetic context of isolates from 26 different nests during dry and rainy seasons.

In Chapters 4 we present results about our search of the *Escovopsis* pathogen in association with *C. minutus* nest components. As we mentioned earlier, this pathogen has not been previously isolated from any yeast agriculture ant. Because no one has looked in detail for the pathogen we decided to use a targeted approach to describe and analyze the fungal community associated with yeast agriculture. In this chapter we describe the fungal community associated with the yeast cultivar garden and the refuse material in the nest.

In all of the chapters we analyze the results in detail and compare them to the fungus-growing ant symbiosis model, phylogeny and the actual knowledge about yeast agriculture ants.

2. *CYPHOMYRMEX MINUTUS* AND ITS YEAST CULTIVAR IN PUERTO RICO

2.1. INTRODUCTION

Cyphomyrmex is a genus of Lower Attini ants that lives in the Neotropics. These ants also cultivate fungi of the Leucocoprinae family that are transmitted vertically by the new queen. *Cyphomyrmex* nests are more often found in the costal zones of Central and North America, the Bahamas and Caribbean islands (Wheeler 1908, Weber 1972). Today, 39 described species are recognized (AntWeb 2012). *Cyphomyrmex* are the smallest Attini ants measuring 1.7-3 mm (Weber 1958). They have a dull and not very sculpted body, move slowly and the head frontal lobes are broad in comparison with other Attini ants (Snelling and Longino 1992). The difference between the coloration of the workers is gradually between brown tones through the whole exoskeleton. The ant workers present a variation in color over time. Younger workers are lighter than older ones (Weber 1972). Color variation is also present at different nests and geographical locations, which can be useful to describe species. *Cyphomyrmex* ants create their nest with a combination of soil, leaf litter, wood and rock. Unlike higher Attini, this genus does not create complex chambers (Weber 1958, Currie 2001a). Instead *Cyphomyrmex* nests are small with only one chamber that is the home of one reproductive queen and less than 200 monomorphic workers (Weber 1958, Snelling and Longino 1992, Mueller 2001, Mehdiabadi and Shultz 2010). In general, *Cyphomyrmex* ants use caterpillars, insect feces and other organic matter to create the substrate for the cultivar (Weber 1958). The *Leucocoprinae* fungi secrete digestive enzymes in to the substrate provided by the Attini ant to degradate the organic matter. Insect corpses cannot be degraded by the cultivar and the ants removed them to the refuse material later (Mueller 2001). Free-living Leucocoprinae close relatives can be found in leaf litter nearby *Cyphomyrmex* nests indicating a recent acquisition of the cultivar by the ants. More detailed studies suggest that

vertical transmission of the cultivar by the new queen have alternated over evolutionary time at least in two horizontal transmission events (Chapella et al. 1994, Mueller 2002, Gerardo et al. 2004). On the other hand, the cultivar generation spam is considerably shorter than the ants. This implies that the cultivar evolved faster than the ants themselves (Chapella 1994, Mueller 2002).

Cyphomyrmex is divided into three different groups (*muelleri*, *stiagatus* and *rimosus* groups) based on phylogenetic analysis of the ant, the fungus clade they cultivate and their agricultural practice (Kempf 1964, Kempf 1966, Snelling and Longino 1992, Gerardo et al. 2004, Shultz and Brady 2008, Mikheyev et al. 2010). Members of the *stiagatus* and *muelleri* groups practice lower agriculture. These ants cultivate Leucocoprinae fungi in a multicellular mycelial phase; the main difference between these two groups is their phylogenetic relationship (Brady and Shultz 2008, Mehdiabadi and Shultz 2010). The most studied species of the *stiagatus* group are: *C. stiagatus, C. faunulus and C. morschi*. The *muelleri* group representative species are: *C. muelleri, C. costatu*s and *C. longiscapus.*
The *rimosus* group includes all the yeast agriculture Attini. The ant species of this group have the ability to maintain the cultivar as a unicellular yeast cluster (2002, Shultz and Brady 2008). All the yeast agriculture Attini cultivars are in the monophylogenetic clade G3 (Chapela 1994, Gerardo et al. 2006). The most common species are: *C. minutus, C. rimosus, C. salvini and C. cornutus*.

The cultivar is a small yellowish yeast cluster that usually measures approximately 0.5mm in diameter that the ants maintain as an irregular rod shape (Snelling and Longino 1992). The pathogen *Escovopsis* has not been found in association with yeast cultivars. *Cyphomyrmex*

minutus was the first species of the genus to be identified by Myrn in 1882 in the Caribbean island of Cuba. In 1907, Wheeler identified this species in Puerto Rico. *Cyphomyrmex* has also been found in a 20 million of years amber fossils from the Dominican Republic over placing this specie in the Greater Antilles between the late Oligocene period and early Miocene (Wilson 1985).

In general, little information about yeast agriculture or for C. *minutus* in particular is available. At the moment, most of the information about this ant is extrapolated from other Attini ants. Besides the above-mentioned information we know that *C. minutus* cultivars from different geographical locations have significant genotypic differences (Mueller 1998b). The cultivar from *C. minutus* (Florida, US) has been isolated and identified in a multicellular phase as *Tyridiomyces formicarum* (Wheeler 1907, Wang et al. 1998). This appears to be the same species of fungi identified from other *rimosus* species (Snelling and Longino 1992). This cultivar produces secondary metabolites (dikertopiperazines) that have antifungal effects over *Saccharomyces cervisiae* and three different human pathogenic strains of *Candida albicans* (Wang et al. 1998). However, the antifungal activity has not been tested in other fungal organisms like *Escovopsis* or proven to be a metabolite that the cultivar produces in the yeast phase. The main purpose of this form of agricultural practice is unknown, but 20-25 million years of coevolution history between the ant and the yeast cultivar and the possible absence of the pathogen suggest an important adaptation (Shultz and Brady 2008, Mehdiabadi and Shultz 2010).

In this study we want to identify and describe in detail *Cyphomyrmex minutus* and its yeast cultivar in Puerto Rico. We present here a multiphasic study of the ant and its cultivar that includes morphology, behavior and genetics.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection

Cyphomyrmex minutus nests were sampled from Cambalache Tropical Forest at Arecibo, Puerto Rico (+18.397803° N, -66.590087°O) during the rainy season 2010 and dry season 2011. We collected a total of 26 different nests for which we gathered data on nest temperature and soil pH. All samples were collected and transported under strict aseptic conditions using flamed-sterilized forceps and sterile containers. In the laboratory, we transferred the nest material into separate petri dishes with damped cotton creating artificial nests. Some samples were used immediately for microbial isolation, while others were allowed to stabilize and used within a 5 days period.

2.2.2. Ant identification and morphological description

To identify and observe the behavior of the ant we used a stereoscope (Olympus SZ2-ILST). The ant was identified using more recently taxonomic keys published by Snelling and Longino (1992).

2.2.3. Description of the cultivar under natural and laboratory conditions

For the initial description of the yeast clusters in the nest we prepared slides of the cultivar and fixed them with lactophenol cotton blue (0.5% w/v) approximately 18 hours after setting the artificial nest in the laboratory. The artificial laboratory nests were prepared with a sterile petri dish with humid cotton and maintained at 25°C in total darkness. Then, we selected three

different yeast cultivar clusters and fixed them with 2.5% gluteraldehyde for 24 hour at 4°C in a 1.5mL microcentrifuge tube. We washed the samples three times with phosphate buffer [0.1 M]. Samples were dehydrated using serial ethanol washes (10%-100%) for15 minutes each. Every time we changed liquids we centrifuge the samples for 30 seconds at 300 rpm. Dehydration was completed by critical point drying for 30 minutes. Dried samples were covered with gold/palladium to allow electron conductivity. Using the scanning electron microscope (SEM) we observed the yeast clusters (De Nollin and Borgers 1975, Gabriel 1982).

2.2.4. Identification of the cultivar by culture independent methods

2.2.4.1. DNA isolation

We selected three cultivar yeast pellets per nest from 17 artificial nests to perform total DNA extraction with Cetyl-trimethyl ammonium bromide (CTAB) modified protocol (Mueller et al. 1998a, Vo et al. 2009). We macerated the yeast clusters with a pestle in 1.5 mL tubes with CTAB. To disrupt the cell we changed temperature from 65°C to -80°C for 10 minutes each time and repeated the process 3 times. Later we treated the samples with chloroform followed by isopropanol and 100% ethanol washes. Samples were dried and then resuspended with TE 1:10 buffer and stored at -20°C.

2.2.4.2. Characterization of the yeast cultivar by Amplification and Sequencing of the D1/D2 region of the 28S rDNA gene

Amplification of the D1/D2 region of the 28s rDNA ene was carried out using approximately 40ng of DNA template in 50µL reactions that included: 0.8x PCR buffer, 2.5nM $MgCL_{2,}$ 0.6µM of each primer, 0.16mM dNTPs and 0.15 µL Taq polymerase per reaction. The selected primers for PCR and sequence were NL-l Forward (5'

GCATATCAATAAGCGGAGGAAAAG-3´) and NL-4 Reverse (5'GGTCCGTGTTTCAAGACGG-3´) (O'Donnell 1993). PCR parameters used: 95°C 3´, 95°C 45´´, 51. °C 45´´, 72°C 1´3´´ 72°C during 30 cycles. Sequencing was performed at the High-Throughput Genomics Unit of the University of Washington, Seattle, WA. We used 10ng/µL DNA amplification product for the reaction. Sequences were analyzed using Sequencher 3.0 (Gene Codes, Ann Arbor, MI) and Mega 5 (Tamura et al. 2011) programs. GenBank searches with BLASTn were performed to identify the cultivar.

2.3. RESULTS

2.3.1. Ant identification and morphological description

In the forest, nest soil presented a temperature of 24.7°C during the rainy season and 23°C in the dry season. Soil pH at the nest was 8.1 at both sampling times. The nests were small and organized in only one chamber as previously described for the species (Wheeler 1908, Weber 1958 and 1972). We observed delimited zones were the ants organize the different component of the nest. The queen was kept apart from the rest of the nest. The brood was kept close to the cultivar and protected by a group of workers. The cultivars were composed of round, white to yellow masses of yeast clusters that measure at least 0.5mm in diameter. We also observed that the ants maintain the cultivar over plant, insect corpses and feces and other unidentified organic materials. All the cultivar pellets look healthy, without any sign of infection under both natural and laboratory conditions. Workers did not present a white cover on their exoskeleton (Figure 2.1 A). The ants weed out, rearrange and manage the pellets using their antennae and frontal legs.

When disrupted, the ants move brood and the cultivar to a deeper location in the nest, while the rest of the workers just used a narcoleptic behavior to camouflage with the soil and the leaf

litter. Under laboratory conditions, we observed fast rearrangements of the artificial nest into zones. Light triggered an immediate emergency protection behavior. The ants move the cultivar and the brood to one side of the nest and the refuse organic material to the other. The refuse materials are black masses of organic material that the ants create and reorganize constantly.

Cyphomyrmex minutus was identified using Snelling and Longino (1992) taxonomic keys for Hymenoptera: Formicidae: Attini ants as follows:

The head width is less than 0.56mm (Figure 2.1 B). This ant presents a preocular curved mesally carina in front of the eye. The posterior-lateral limits of the scrobe are marked by another carina that is arising from the occipital corner and to the eyes. Lateral pronotal tubercles are present (Figure 2.1 C). The mesonotal tubercles are elevated and conical. The texture of the mesosoma is granulose. All body hairs are fine in comparison with other species; many of them are dentiform. The median basal groove of the first gastral tergum is short almost indistinctive (Figure 2.1 C).

Figure 2.1: *Cyphomyrmex minutus* collected in Cambalache Tropical Forest. (A) *C. minutus* ants with the yeast pellets cultivar in artificial nest. (B) Frontal view of the ant. (C) Lateral view of the ant.

2.3.2. Description of the cultivar under natural and laboratory conditions

The cultivar is a *Leucocoprinae* fungus that is maintains as an irregular rounded yeast cluster by *Cyphomyrmex minutus* (Figure 2.2A). Under laboratory conditions, the cultivar in the artificial nest starts presenting hyphal growth. After 18 hours, the cluster has a combination of yeast cells and pseudo-hyphae growing in the external areas of the pellet as observed under SEM (Figure 2.2).

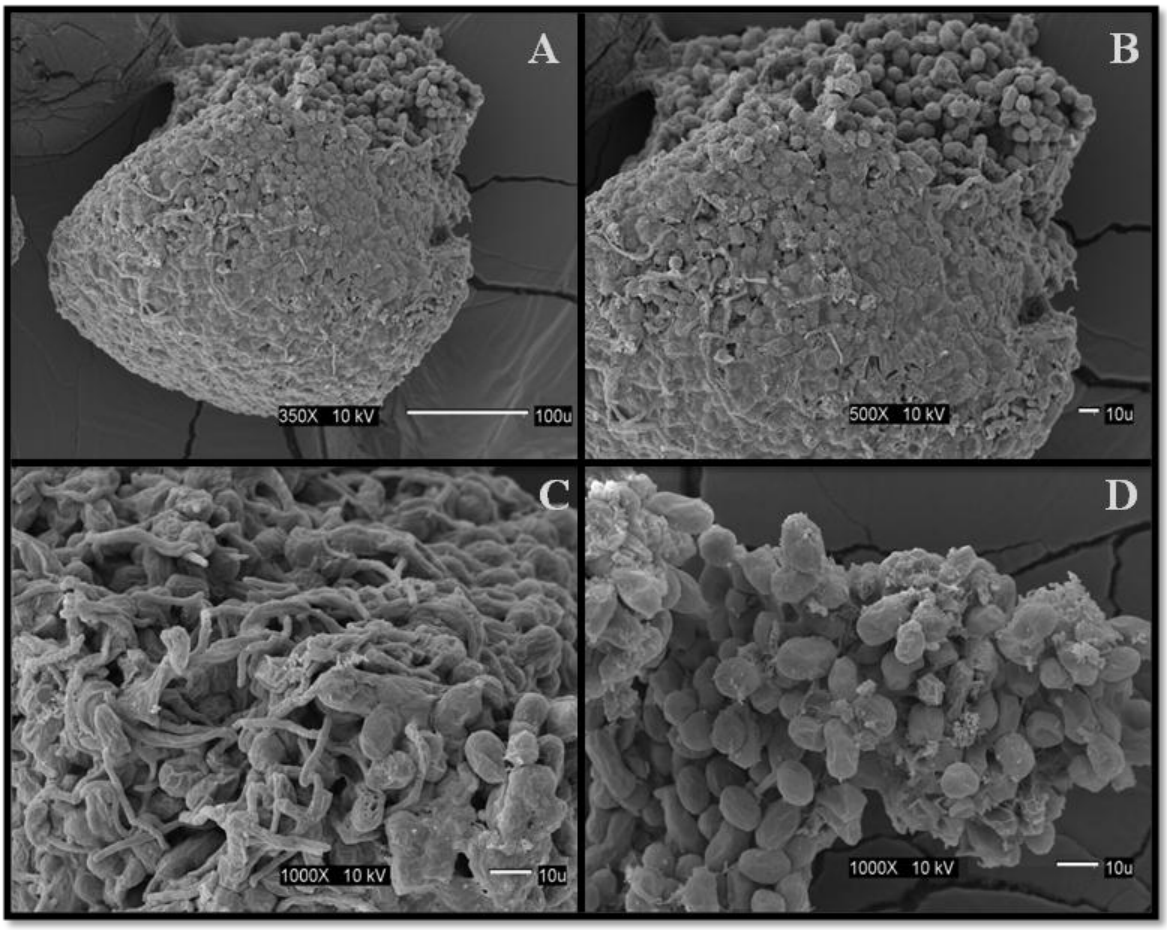

Figure 2.2: Yeast cultivar of *Cyphomyrmex minutus* from Cambalache Forest fixed (18 after collection). (A-D). Partially disrupted yeast cluster created by the ants at (A) 350x and (B) 500x. (C) A close up (1000x) shows zones with hyphal growth and (D) ellipsoidal yeast cells.

In order to understand the progression of the cultivar over time under laboratory conditions, we observed the cultivar for a period of 5 days (Figure 2.3). At the beginning, we observed yeast round cells consistent with previous description of yeast agriculture in *Cyphomyrmex* species (Figure 2.3 A-C). However, after 24 hours we observed cell elongation and pseudohyphal development (Figure 4D). This development persists after 72 hours (Figure 2.3E) and continues progressively for the next few days. After day 5, the cluster maintains the same appearance to the naked eye, but under the microscope we observed a mixture of yeast and pseudohyphal growth (Figure 2.3F), the latter being the predominant growth form.

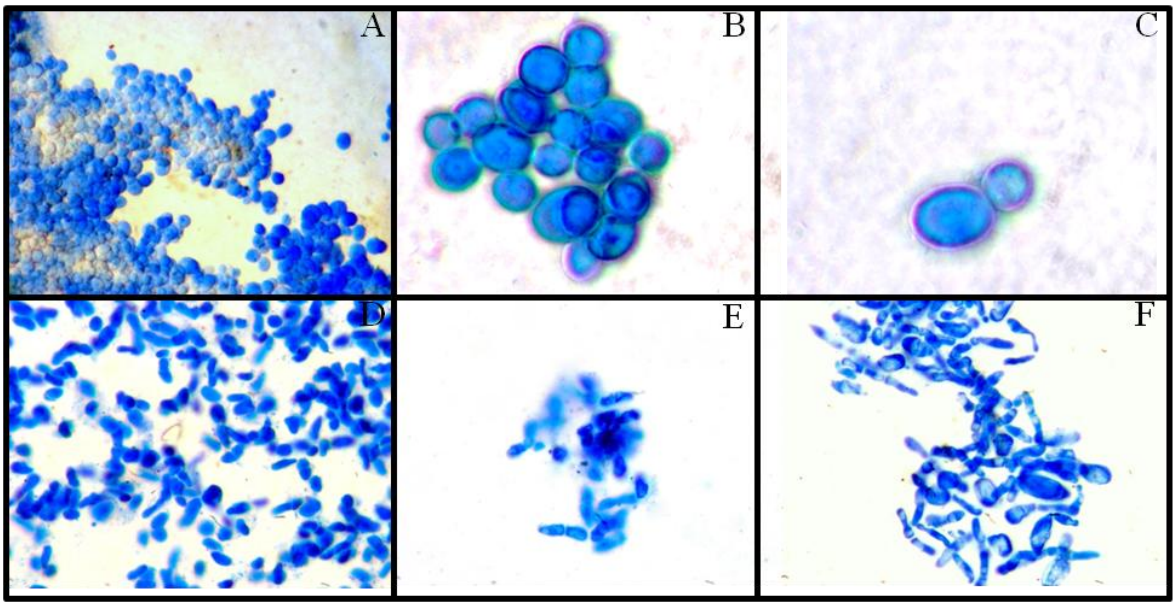

Figure 2.3: Yeast cultivar stained with lactophenol cotton blue under laboratory conditions at 24 hours (A-C). (A) Disrupted yeast pellet (4x), (B) yeast cells (20x), (C) budding yeast (40x). (D**)** Yeast cultivar growth after 24 hours (20x), (D) after 72 hours (20x) and (F) after five days (20x).

2.3.3. Identification of the cultivar by culture-independent methods

The cultivar was identified as Leucocoprinaseus fungi similar to other *C. minutus* cultivars. We compared *C. minutus* symbiont from Puerto Rico to other fungus-growing ant cultivars from all agriculture practices (Figure 2.4). Our cultivar showed a close relationship with *C. minutus* symbiont 950106-03 from Trinidad with 98% similarity.

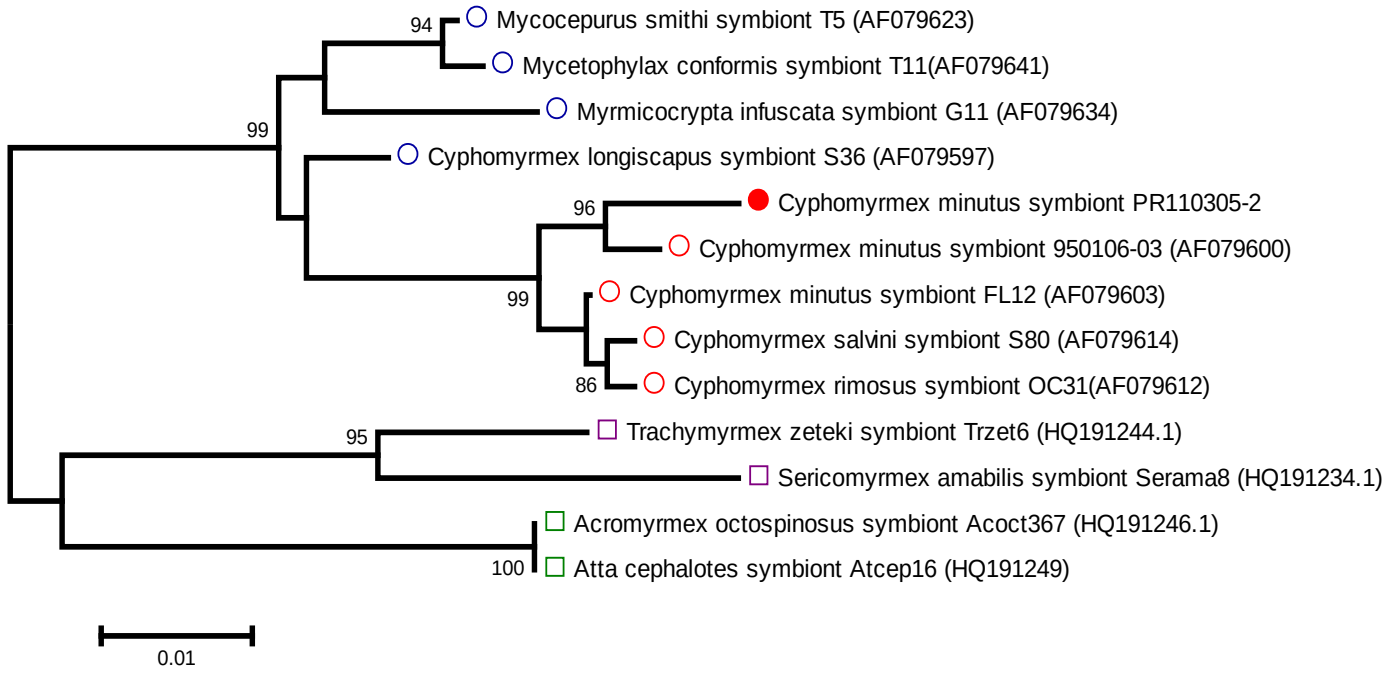

Figure 2.4: Maximum Likelihood (ML) phylogeny of fungus-growing ants cultivars based on partial 28S rDNA gene. We compared the consensus sequence of *Cyphomyrmex minutus* cultivar isolated from Cambalache Tropical Forest in Puerto Rico (red full circle) with other fungus-growing ant cultivars. The Lower Attini cultivars are represented with circles and Higher Attini with squares. The colors represent the type of agriculture that the source ant practices: lower agriculture in blue, yeast agriculture in red, domesticated higher agriculture in purple and leaf-cutter agriculture in green. The sequences for comparison were retrieved from GenBank database using BLASTn. The scale bar corresponds to 0.01 substitutions per site and bootstrap support after 5,000 replicates.

2.4. Discussion and Conclusions

Characteristics of *Cyphomyrmex minutus* ants from Puerto Rico are consistent with other *C. minutus* even when this ant presents a lighter coloration of the exoskeleton in comparison with other populations (AntWeb 2012). Yeast agriculture is the main activity of the colony including substrate acquisition, protection, rearrangement, weeding and grooming. When we try to replicate the appropriate conditions (temperature, humidity and darkness) for the nest in the laboratory, the cultivar cluster started developing pseudohyphae in less than 24 hours. *Cyphomyrmex minutus* cultivar presented a pleomorphisim, which indicates that this fungus can grow as yeast and as mycelium depending on environmental conditions (Mueller 2001).

After the fifth day, 90% of the artificial nests were dead. Based on our results, we understand that the yeast phase of the cultivar represents a signal of a healthy nest. In addition, the absent of any visual sign of infection and the observed ant behavior support the idea that the yeast phase of the cultivar serves as a defense mechanism for the nest against fungal pathogens. Using microscopy and 28S DNA sequencing we were able to identify the cultivar as a Leucocoprinae fungus closely related to a *C. minutus* cultivar from Trinidad and Tobago located it in the G3 clade with other yeast agriculture cultivars (Mueller 2001). The next close relative is a *C. minutus* cultivar from Florida (US) also in the same clade. Taking in consideration previous reports, we observed marked differences between our *C. minutus* cultivar sequences and other yeast cultivars from the *rimosus* group. The data is consistent with multiple events of horizontal acquisition, hence showing some geographical separation between continental and Caribbean strains. Unfortunately we did not have access to previously described mycelia (*Tyridiomyces formicarum*) associated with *C. minutus* in Florida or its sequence for comparison.

The identification of the ant and the cultivar from Puerto Rico provides new information about fungus-growing ants in the Caribbean. In addition, the identification of the ant and the cultivar, their behavior and documentation provide modern information about the yeast agriculture practice, which is the most understudied system in the Attini. This study sets the bases to identify, describe and understand other microorganisms involved in this fungus-growing ant symbiosis and their interactions.

3. ACTINOBACTERIA ASSOCIATED WITH *C. MINUTUS* EXOSKELETON AND ITS CULTIVAR

3.1. INTRODUCTION

Attini ants have 50 million years of coevolution history with Leucocoprinae fungi that serve as their main source of food (Currie 2001a). Attini ants provide the cultivar with protection, growth conditions and substrate. The same interaction has been reported in 5 different agriculture practices: lower agriculture, coral fungi agriculture, yeast agriculture, higher domesticated agriculture and leaf cutter agriculture grouping over 230 different ant species (Shultz and Brady 2008, Mehdiabadi and Shultz 2010). The ant transmits the cultivar vertically from one nest to the new one by the queen during the nuptial flight. In the nest cultivar propagation appears to be asexual, which can significantly decrease genetic variability in comparison to free-living sexually reproducing counterparts (Currie 2001a). On other hand, the Attini nests are in the soil, leaf litter, wood or rocks (Wheeler 1907, Currie 2001a), which are far from being axenic environments for the cultivar. Clonally propagation and asexual reproduction in addition to ant nest environmental conditions make the cultivar vulnerable to mycopathogens. Nests infected with the specific pathogen *Escovopsis* have a significant reduction in fitness and greater potential death (Currie et al. 2006).

To understand how the ants protect their main source of foods multiple defense strategies such as weeding, farming, nest material rearrangement, nest chamber organization, refuse material collection, glandular and cultivar antifungal secretions and, association with antibiotic producing actinobacteria have been explored (Currie 2001a, Currie et al. 2006, Shultz and Brady 2008, Mehdiabadi and Shultz 2010). Some of these strategies are not constant across all agricultural practices or have not been studied in detail (Shultz and Brady 2008, Mehdiabadi and Shultz 2010).

Actinobacteria associates have been described as an important defense mechanism for Attini agricultural practices (Currie et al. 2003, Currie et al. 2006). The phylum Actinobacteria is a group of Gram positive bacteria with high G+C content (>55 mol% in genomic DNA) (Champness 2000, Gao and Gupta 2012). They are cosmopolitan organisms that can live in water, deep-sea and extreme environments, but most of the studied species are isolated from soils. Over 300 different genera are members of this phylum with an enormous diversity of morphology, physiology and metabolic capabilities. Low divergence of 16S rDNA gene sequences between members of the same genus, e.g. *Frankia* species are often between 97.8%-98.9% similar, indicates a very close relationship between species (Gao and Gupta, 2012). Additional gene information is needed to resolve a phylogeny of closely related genera of Actinobacteria to species. Currently, 16S rDNA is still in use as the preferred method to study Actinobacteria phylogeny, but several markers have been proposed as alternatives to further resolve species relationships. The most promising ones are the Conserved Signature Proteins (CSP) and Conserved Signature Indels (CSI). These proteins can be used as markers because they are part of the ribosomal protein complex, RNA and DNA polymerases and key metabolic enzymes. In addition, they are unique to particular groups of Actinobacteria (Gao and Gupta, 2012).

The phylum Actinobacteria was recently divided into 6 different classes: *Actinobacteria, Acidimicrobiia, Rubrobacteria, Coriobacteriia, Nitriliruptoria* and *Thermoleophilia* (Gao and Gupta 2012). *Actinobacteria* is the biggest class and contains the most common and well-studied genera such as: *Actinomyces*, *Mycobacterium, Rhodococcus*, *Nocardia, Pseudonocardia* and *Streptomyces* (Garrity et al. 2004). The latter is the most common,

frequently isolated and well-studied genus (Brenner et al. 2005). The Actinobacteria were classified in the past as fungi (*Actinomycetes*, name still in use as a synonym) because of their macroscopic and microscopic morphology in combination with an atypical reproduction cycle (Angert 2005, Brenner et al. 2005, Del Sol 2007).

Actinobacteria morphology can be described as a filamentous bacillus with special lipids in the cell membrane (Brenner et al. 2005). Many Actinobacteria genera are important secondary metabolite producers such as antibacterial, antifungal, antitumor, antiviral, herbicidal, insecticidal and immunosuppressive compounds. Also some species have the capacity of complex polysaccharide degradation (Angert 2000, Brenner et al. 2005). In 2000, over 12,000 different antibiotics were identified from natural sources, 70% derived from Actinobacteria and 55% from the genus *Streptomyces* (Angert 2000).

The mutualism between the Actinobacteria and Attini ants has a long history of coevolution (Currie et al. 1999a). The funder new queen carries the Actinobacteria symbiont on its exoskeleton to the new nest (Currie et al. 1999a). Attini from the lower agriculture genera *Mycocepurus* and *Cyphomyrmex* as well as the higher agriculture *Trachymyrmex* and *Acromyrmex* have visible Actinobacteria on their propleural plates (Currie et al. 2006). Some species present Actinobacteria covering other areas of the exoskeleton like: head, thorax, abdomen and legs (Currie et al. 2006). *Atta* (leaf cutter agriculture) species do not show any visible Actinobacteria growth on their exoskeleton; meanwhile yeast agriculture *Cyphomyrmex* species have not been observed in detail.

On the propleural plates the Attini have elaborated cuticular crypts associated with exocrine glands. Located in the crypts are foveae that host the Actinobacteria symbionts. The glands at

the inner surface of the propleural plates are connected by duct and glandular cells to the cuticle and opened to the fovea putatively providing nutrients to the symbionts (Currie et al. 2006). In addition, the ants also have metaplural cuticular exocrine glands, which secret substances considered as broad-spectrum antimicrobials (Bot et al. 2002, Fernandez-Marin et al. 2006).

Original observations of Attini ants described a distinctive white cover over the exoskeleton that further studies identified as Actinobacteria growing on the exoskeleton (Weber 1972, Currie 2001a). Based on the cell wall chemical composition and morphology of the isolated bacteria from the exoskeleton it was identified as *Streptomyces* (Currie et al. 1999b, Currie 2001b). Further studies that included sequence analysis of the Actinobacteria isolated from *Acromyrmex, Trachymyrmex* and *Apterostigma* indicated that the most prevalent Actinobacteria was *Pseudonocardia* (Cafaro and Currie 2005). Later a culture independent analysis of the Actinobacteria showed a high prevalence of other two genera of Actinobacteria: *Streptomyces* and *Amycolatopsis* from laboratory nests of *Trachymyrmex*, *Serichomyrmex* and *Cyphomyrmex* (non-yeast agriculture species) (Sen et al. 2009). This study demonstrated coexistence of different genera in association with Attini ants. *Streptomyces* and *Amycolatopsis* isolates from the same samples affect potential nest pathogens (including *Escovopsis*) growth in at least 56.3 -72.7% of the cases (Sen et al. 2009). Some discrepancies in the identification of the Actinobacteria indicate that more studies are needed to clear this matter; in the meantime we use all the information available to understand the Attini symbiosis system. *Pseudonocardia* and *Amycolatopsis* are two genera from the same family (Pseudonocardineae), while *Streptomyces* belongs to a different suborder (Streptomycetaceae) (Garrity 2004, Brenner 2005). These 3 different genera (*Streptomyces,*

Pseudonocardia and *Amycolapsis*) have been isolated from different fungus-growing ants using different methods and have antibiotic production potential (Currie et al. 1999a, Currie et al. 2006, Cafaro and Currie 2005, Sen et al. 2009).

In this mutualism the ants get protection for the cultivar against pathogens, as a reward the ants serve as a vector for the bacteria and also provide protection and nutrients (Currie et al. 2006, Poulsen et al. 2010). These interactions support the idea of a complex mechanism of cultivar defense that includes the propagation and use of antibiotic producing microorganisms to control pathogens. The specialized structures present in the ant exoskeleton indicate a long and strong interaction of these four symbionts: the ants, the cultivar, the cultivar pathogen and the Actinobacteria (Currie et al. 1999a, Currie et al. 1999b).

In the specific case of the members of the *Cyphomyrmex rimosus* group there is no evidence about this type of protective mechanism (including the Actinobacteria symbiont). Over the years many assumptions and generalizations have been made in order to understand the fungus-growing ant agricultural practices in general (Sen et al. 2009, Mehdiabadi and Shultz 2010). The only certain thing we know about the *rimosus* group symbiosis and their defense mechanism is that the ant cultivates Leucocoprinae fungi as yeast (Weber 1958). There is no study about the apparent absence of the specific pathogen *Escovopsis* and any defenses that can contribute to the health of the cultivar. Because there are many unanswered questions about yeast agriculture we want to describe the Actinobacteria community associated with *Cyphomyrmex minutus* exoskeleton and its cultivar in detail. We also studied the propleural plate structure and explore its potential as Actinobacteria hosting structure as described for other Attini species.

3.2. MATERIALS AND METHODS

3.2.1. SAMPLE SELECTION

Samples from stabilized artificial *C. minutus* nests were processed in the laboratory to isolate Actinobacteria the same day of sampling. We selected 3 cultivar pellets and 3 ants form each nest. We collected samples in two periods, 2010 rainy season and 2011 dry season at Cambalache Tropical Forest.

3.2.2. ACTINOBACTERIA IN THE EXOSKELETON AND THE PROPLEURAL PLATES

We treated samples differently if we wanted to observe the propleural plates and exoskeleton structures or if we wanted to observe associated microorganisms. For the first treatment we fixed *C. minutus* ants in 2.5% gluteraldehyde during 24 hour at 4°C in a 1.5mL microcentrifuge tubes for SEM analysis. We repeated 3 washes with phosphate buffer [0.1 M] for samples. We did not fix the second group. Then we dehydrated using serial 10% increase ethanol washes for the structure samples and serial 5% increase ethanol washes for the microorganisms. All samples were decanted at room temperature for 15-30 minutes. Later we use a critical point dryer for 30 minutes. All dried samples were covered with a gold/palladium to allow electron conductivity. Using the Scanning Electron Microscope (SEM) (JEOL JSM-5410LV) from the Microscopy Center of the Biology Department we observed both samples (De Nollin and Borgers 1975, Gabriel 1982).

3.2.3. ACTINOBACTERIA PURE CULTURE ISOLATION

Separately, the ants and the cultivar pellets were washed, macerated and mixed with vortex in 1.5mL microcentrifuge tubes with 900µL of 0.7% NaCl. In triplicates, we inoculated 300 µL of each wash in Chitin media plates (Chitin 3g, K_2HPO_4, 0.575g, $MgSO_4$ x $7H_2O$, 0.375g, KH_2PO_4, 0.275g, $FeSO_4$ x $7H_2O$ 0.0075g, $MnCl_2$ x $4H_2O$ 0.00075g, $ZnSO_4$ x $7H_2O$ 0.00075g and agar 15g in a final volume of 750mL of ddH_2O). To avoid fungal growth we supplemented the media with Nystatin (0.02g/ml of DSMO) and Cyclohexamide (0.05g/L). We spread the solution and incubated for 3-4 weeks at 25°C until growth was evident. Colony selection and further purification was performed every week during the incubation period. Colony transfers were made into Yeast and Malt Extract Agar (YMEA) (Yeast Extract 4g, Malt Extract 10g, Dextrose 4g, and Agar 20g per 1L of dH_2O) with antimicotics (Nystatin and Cyclohexamide). Samples in YMEA were incubated at 25°C until we observed growth. Multiple transfers were needed to obtain pure colonies. The time of incubation depended on the samples (3 days - 4 weeks). All samples were preliminarily classified using morphology and Gram staining (Brenner et al. 2005).

3.2.4. DNA extraction and 16S rDNA gene amplification

We extracted total DNA from all isolates in pure culture with the Cetyl-trimethyl ammonium bromide (CTAB) modified protocol (Mueller et al. 1998, Vo et al. 2009). We macerated the cells with a pestle in 1.5 mL tubes with CTAB. To disrupt the cell wall, we subjected the samples to three cycles of freeze (-80°C) - thawing (65°C). Later the samples were treated

with chloroform followed by isopropanol and 100% ethanol washes. Samples were dried and then resuspended with TE 1:10 buffer and preserved at -20°C.

Amplification of the 16s rDNA gene was done using approximately 40ng of DNA template was used for amplification in 50µL reactions which included: 0.8x PCR buffer, 2.5nm $MgCL_{2,}$ 0.3µ M of each primer 0.16mM dNTps and 5U Taq polymerase per reaction. We used the following thermal parameters: 95°C 3´, 95°C 45´´, 52°C 45´´, 72°C 1´3´´and 72°C for 30 cycles. We used universal bacterial primers 27F (5´AGA GTT TGA TCM TGG CTC AG) and 1492R (5´TAC GGH TAC CTT GTT ACG ACT T) (Lane 1991) to amplify the 16S rDNA gene. The fragment of approximately 1470bp was sequenced at the High-Throughput Genomics Unit of the University of Washington, Seattle, WA. We used 10ng/µL DNA amplification product for the reaction.

3.2.5. Data Analysis

3.2.5.1. Frequency and Diversity index

We calculated the frequency of the isolates in terms of percentage. Also we analyzed the two communities in terms of species diversity and dominance using Simpson (S) and Shannon (H) indices (Shannon and Weaver 1949, Simpson 1949). We used an Excel (Windows office 2007) to calculate both indices using the following formulas:

- The Simpson Index
    - Diversity (S): S=1- D
    - Dominance (D): $D = \sum_{i=1}^{r} \frac{x_i(x_i-1)}{t_0(t_0-1)}$

      where: r = total number of species or taxonomical units observed
      xi= refers to the number of each sample
      t_0= total abundance = $\sum_i^r x_i$

- The Shannon Index
    - Diversity (H): $H = -\sum_{i=1}^{r} p_i \ln p_i$
      where: r = total number of species or taxonomical units observed
      xi= refers to the number of each sample
      t_0= total abundance = $\sum_{i}^{r} x_i$
      p_i= relative frequency = - $\frac{x_i}{t_0}$
    - Eveness (E): $E = H/\ln(r)$

3.2.5.2. Sequence analysis

Sequences of the 16s rDNA gene were edited and analyzed using Sequencer 3.0 (Gene Codes, Ann Arbor, MI) and Mega 5 (Tamura et al. 2011) programs. Ribosomal Data Base (ref) and GenBank searches with BLASTn were performed to identify the closest available sequences. In Mega 5 we used Muscle application to align sequences with the following parameters: Refining alignment, -400 penalty for gap open and -0.01 penalties for gap extension. The phylogenetic tree was created using Mega 5 Neighbor joining analysis with 5,000 pseudoreplicates for bootstrap support and a p-distance model.

3.3. RESULTS

3.3.1. *CYPHOMYRMEX MINUTUS* PROPLEURAL PLATE AND MICROORGANISMS ASSOCIATED WITH THE EXOSKELETON SURFACE

Our initial observations using light microscopy indicate that *C. minutus* possesses propleural plates similar to other non-yeast agriculture members of the genus. In addition, no worker from 26 different nests presented any visible white cover on the exoskeleton. Using scanning electron microscopy (SEM) we studied specific cuticular structures on *C. minutus*. First we identified crypts or foveae at the propleural plates (Figure 3.1 A and B). Plates also presented few microtrichia (hairs-like projection) located around the foveae (Fig 3.1). Foveae are only

located on the propleural plates of the ant close to the head and the frontal legs. The foveae measured less than 10µm in diameter and were scattered around the plates (Figure 3.1 A). Inside the foveae we observed a porous and irregular surface, completely different from the propleural plate surface (Figure 3.1 B). The plate surface and microtrichia were covered with unidentified substances or microorganisms (Figure 3.1C-F). In some cases, a globular coverage was present inside some foveae (black arrow Figure E). Because of the description and size of the globular coverage we suggest that this is consistent with secretion products fixed during sample preparation, but we cannot rule out microbial growth associated with the secretion.

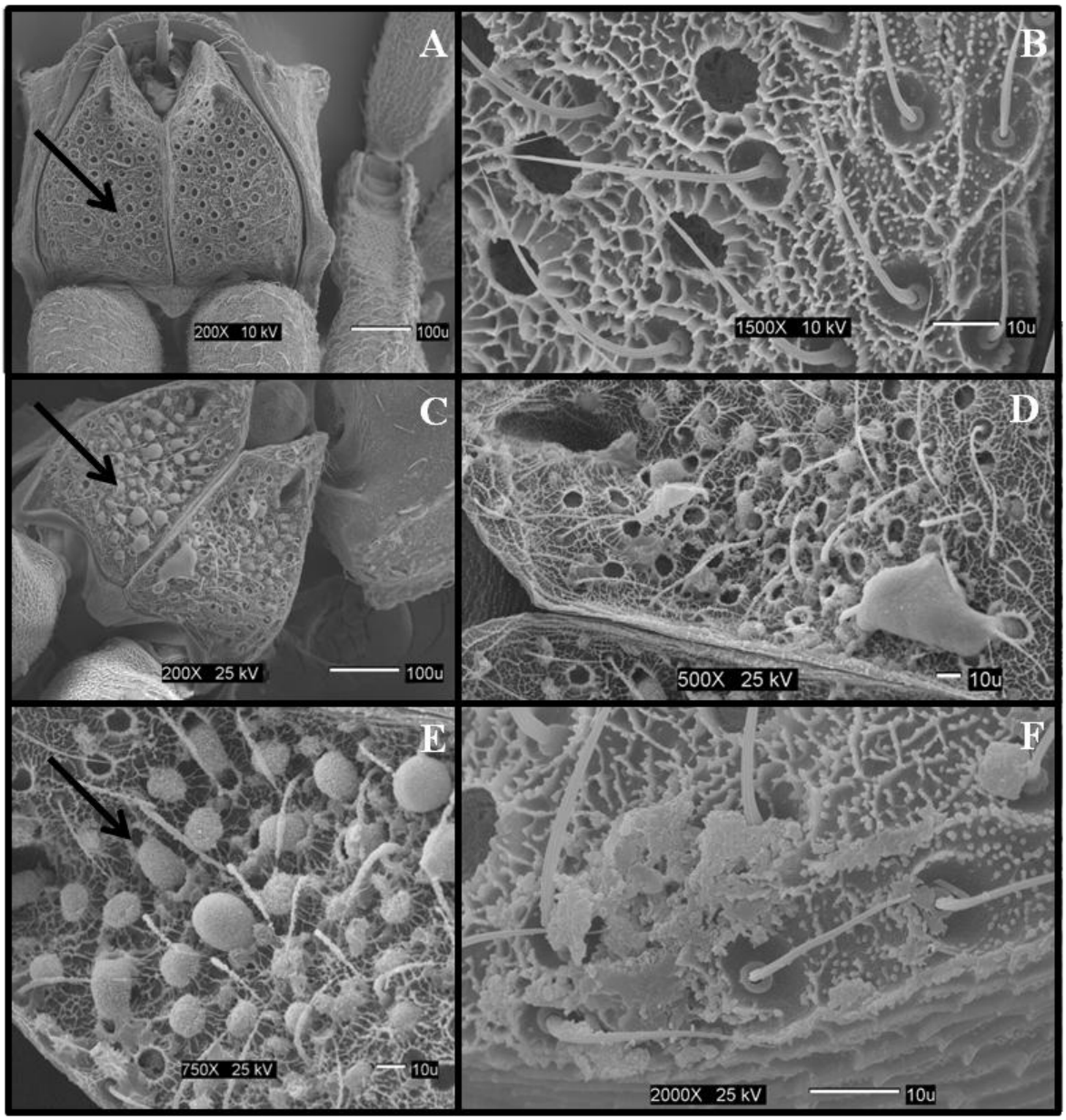

Figure 3.1: SEM pictures of *Cyphomyrmex minutus* propleural plate. (A) Crypts in the propleural plates are show up by the black arrow. (B) A zoom in of the crypts (C) Propleural plate covered with an unidentified substance or microorganism. (D) Close up of the propleural plate showing globular substance coving the surface (black arrow) (F) Accumulation of unidentified cover over the propleural hairs.

In addition to the propleural plates we examined the ant head, thorax and abdomen. The ant head presented pores without microorganisms, hairs and duct cells that cover its surface (Figure 3.2A). The same area in another ant sample presented a conspicuous microbial

coverage over the ant head (Figure 3.2B). The ant frontal legs without fixation presented hair like projections, pores (black arrow on figure 3.2C) and glandular secretary ducts (black arrow on figure 3.2E). The frontal legs surface presented microbial growth on their surface (Figure 3.2D, F-H), which was characterized by mycelium-like growth (Figure 3.2D and G) and visible bacilli close to glandular ducts (black arrow on Figure H).

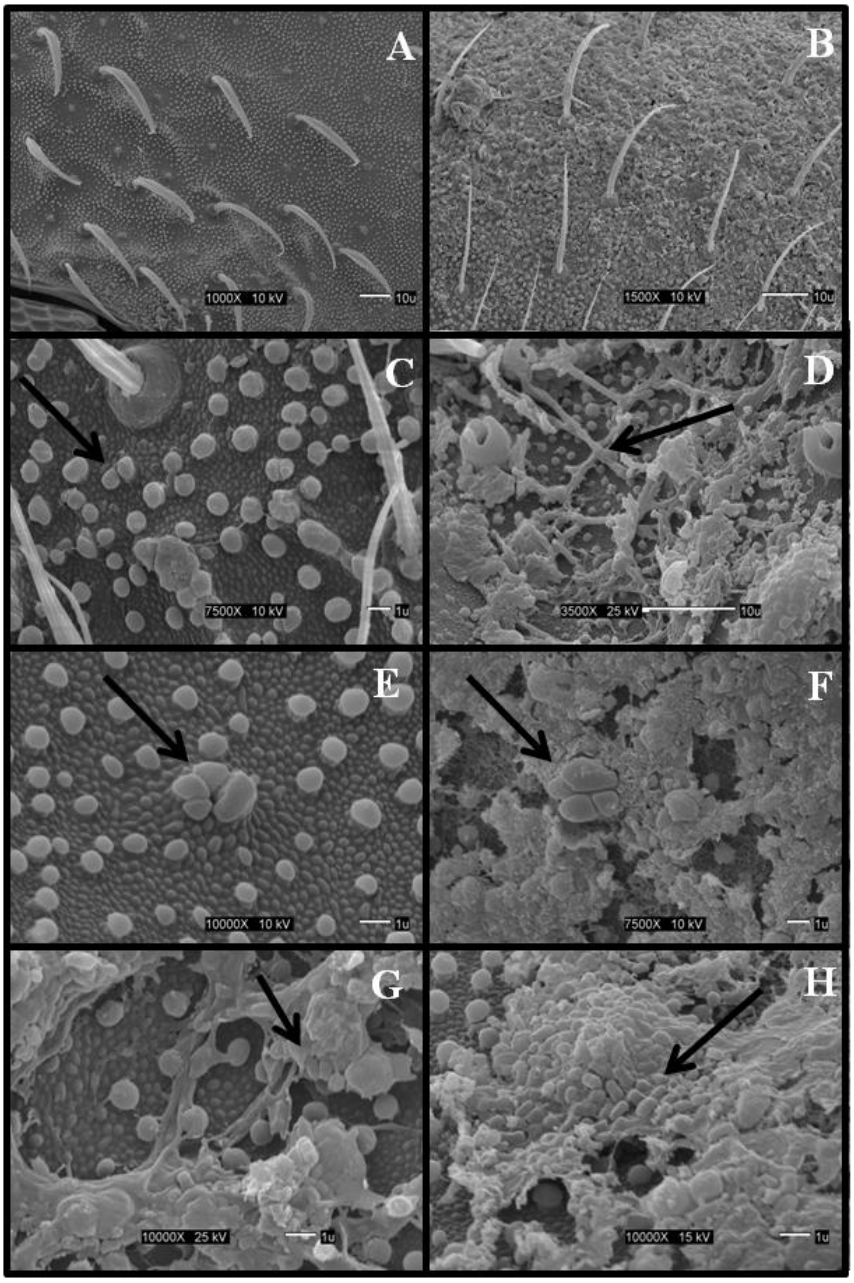

Figure 3.2: SEM microphotographs of different areas of *C. minutus* exoskeleton. Samples in picture A, C and E present only the ant exoskeleton surface. Samples in the pictures B, D, F, G and H were fixed to preserve any biological coverage. (A-B) Ant head close by the ocular aperture (A) the surface with pores, hairs and (B) the same area preserved to observe the microorganisms covering. (C-H) Forelegs close up of the exoskeleton. (D) Possible mycelia growth in the foreleg exoskeleton (arrow). (E) Glandular aperture (arrow) on the exoskeleton. (B) Same structure (arrow) with microbial growth. (G -H) Bacillus type cells (arrow) growing around the glandular aperture.

3.3.2. ACTINOBACTERIA ASSOCIATED WITH THE ANT EXOSKELETON AND THE YEAST CULTIVAR

Actinobacteria present small differences in16S rDNA gene sequences between species, which can be used to identify genera and in some cases established a connection between isolates and well-studied type strains. Type strains are available both at the Ribosomal Data Base Project and GenBank websites (Appendix A). With this information we attempted to describe and classify the isolates associated with *C. minutus* and compare them to other available data from attine ants.

We analyzed and identified 208 different isolates from 26 nests in Cambalache Tropical Forest during the rainy and dry seasons. Using 16S rDNA we identified 5 different Actinobacteria genera. *Nocardia, Rhodococcus, Kitasatospora, Tsukamurella* and *Streptomyces*. The most frequent isolated genus was *Streptomyces*, which represented 93% of all isolates. Other genera were less frequent: *Nocardia* (1%), *Rhodococcus* (2%), *Kitasatospora* (2%) and *Tsukamurella* (2%) (Figure 3.3).

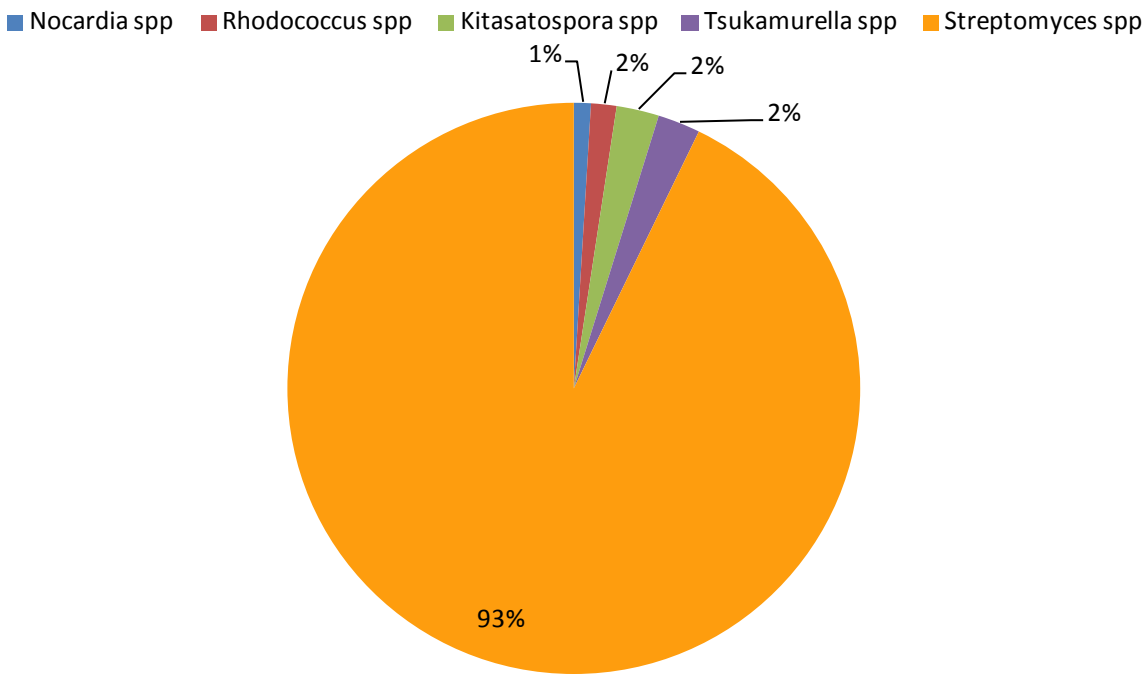

Figure 3.3: Actinobacteria genera isolated from *C. minutus* exoskeleton and its yeast cultivar

The most frequently isolated strain overall was *Streptomyces* sp. 31 (similar to *Streptomyces* sp. 8-1 EU054375.1), which represented 41.83% of all isolates. Other strains of Streptomyces were also fairly abundant, but in smaller proportions: 4.33% *Streptomyces* sp. 9 (similar to *Streptomyces cinereoruber* NR043344.1), 3.85% *Streptomyces* sp. 11 (similar to *Streptomyces exfoliatus* FJ532461.1) and 3.37% *Streptomyces* sp. 23 (similar to *Streptomyces lateritius* GU479442.1). The rest of the isolates (47 potential species) combined represent 46.63% of the total, but each independently has frequencies lower than 3% (Figure 3.4).

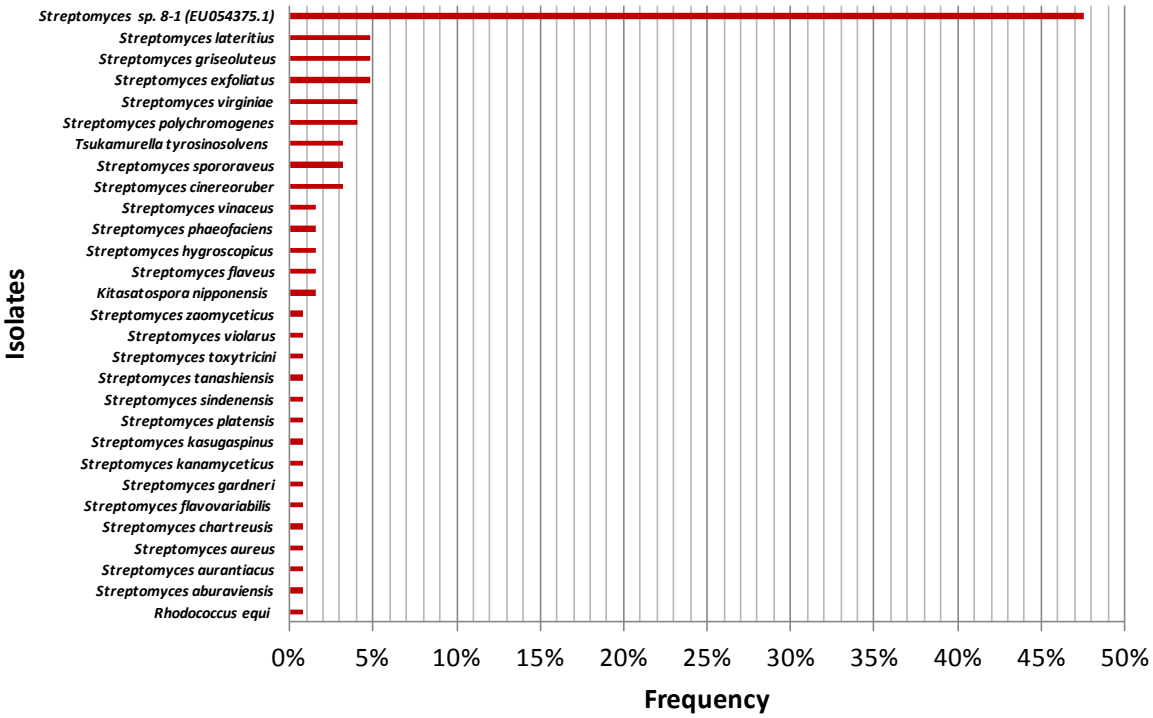

Figure 3.4: Frequency of the Actinobacteria isolates associated with *Cyphomyrmex minutus* exoskeleton.

From the yeast cultivar we identified 36 potential species from 84 different samples. All samples came from ant cultivar derived from 26 different nests of *C. minutus*. We isolated 4 Actinobacteria genera from cultivar washes: *Kitasatospora*, *Nocardia*, *Rhodococcus* and *Streptomyces* (Figure 3.5). Again, *Streptomyces* was the most frequent genus isolated from the samples. The most frequent *Streptomyces* strain was *Streptomyces* sp. 31, similar to *Streptomyces* sp. 8-1 EU054375 (33%). Other frequent isolates were: 6% *Streptomyces* sp. 38 (similar to *Streptomyces yaglinensis* AY882020.1), 6% *Streptomyces* sp. 9 (similar to *Streptomyces cinereoruber* NR043344.1), 4% *Streptomyces* sp. 26 (similar to *Streptomyces phaeogfaciens* HQ607439.1), 4% *Streptomyces* sp. 24 (similar to *Streptomyces omiyaensis* AB184411.1) and 4% *Streptomyces* sp. 22 (similar to *Streptomyces kummingensis*

NR043823.1). The other 30 isolates combined represented 43% of the total. Individually, no strain exceeded 2% in frequency. The complete list of all the isolates appears in Appendix A.

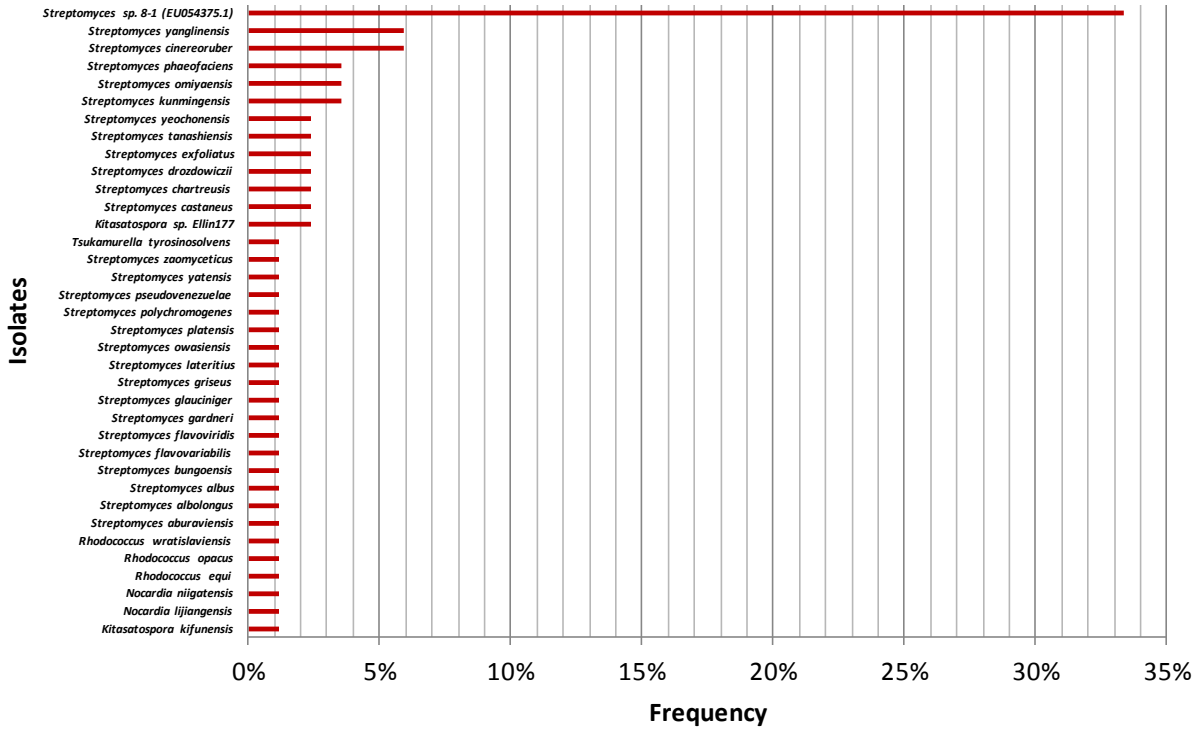

Figure 3.5: Frequency of isolates associated with *Cyphomyrmex minutus* yeast cultivar.

3.3.3. DIVERSITY INDICES

To describe the biodiversity of the cultivable Actinobacteria taxa isolated from *C. minutus* exoskeleton and its yeast cultivar we used the Simpson (S) and the Shannon (H) Indices (Table 3.1). The Simpson Index evaluates the quantity of species in the sample and the richness in each sample (entropy). With the Dominance (D) calculation, it can also evaluate the presence of dominant species over the rest of the population. The Actinobacteria community associated with *C. minutus* exoskeleton (29 different isolates from 124 samples) presented a diversity entropy indicator S_{ant} = 0.0836 and dominance value D_{ant} = 0.9164. In

the case of the Actinobacteria community associated with the yeast cultivar (36 different isolates from 84 samples) using the same indeces we obtained $S_{cultivar} = 0.0192$ for entropy and $D_{cultivar} = 0.9808$ for dominance. The Actinobacteria community from the ant exoskeleton appears to be less entropic in terms of species diversity than the community from the yeast cultivar ($S_{ant} = 0.0836 > S_{cultivar} = 0.0192$). The possibility of dominant species in the community is higher in the case of the Actinobacteria community isolated from the cultivar.

To confirm the results we also analyzed them using the Shannon Index (H). This index evaluates the number of observed individuals for each species. In the case of the Actinobacteria community from the exoskeleton we obtain $H_{ant} = 2.3002$ index value and $H_{cultivar} = 2.8952$ for the Actinobacteria community from the yeast cultivar. Values over 2.0 indicate variety of species in the community diversity. The exoskeleton community seems to be less diverse in terms of species numbers than the yeast cultivar Actinobacteria community ($H_{ant} = 2.3002 < H_{cultivar} = 2.8952$). We also calculated Eveness (E), which indicates how close in species number are the communities. The Eveness indicators for both communities exceed 1, indication that the proportion of members of the each species is similar in both communities (Table 3.1).

Table 3.1: Simpson Index values for the Actinobacteria community isolated from *C. minutus* exoskeleton and its yeast cultivar.

Diversity index	Actinobacteria isolated from	
	Ant exoskeleton	**Ant yeast cultivar**
Simpson Index (S) S=0 low entropy (one species or few) S=1 high entropy (diverse community)	**0.0836**	**0.0192**
Dominance (D=1-S) D=0 all species are equally present D=1 one species dominates the community completely	**0.9164**	**0.9808**
Shannon Index (H) H=0 only one species in the community H>0 more than one species in the community	**2.3002**	**2.8952**
Eveness (E=H/ln(isolates)) E=1 similar proportion of all species in the community E>1 dissimilar proportion of the species in the community, dominant species	**1.5729**	**1.8603**

3.3.4. PHYLOGENETIC RELATIONSHIPS OF ACTINOBACTERIA ISOLATES

We created 16S rDNA phylogenetic trees comparing the relationships between Actinobacteria isolated from the ant exoskeleton and the yeast cultivar to database sequences (Figures 3.6 and 3.7). Two orders in the Class Actinobacteria, Streptomycetales and Corynebacteriales were represented in our samples from both ant exoskeleton and yeast cultivar. The most frequent *Streptomyces* isolate, represented by sequences PR110305M-H214 and -AL610, are closely related to *Streptomyces* sp. 8-1 EU054375, which relates to the type species *S. fulvissimus*.

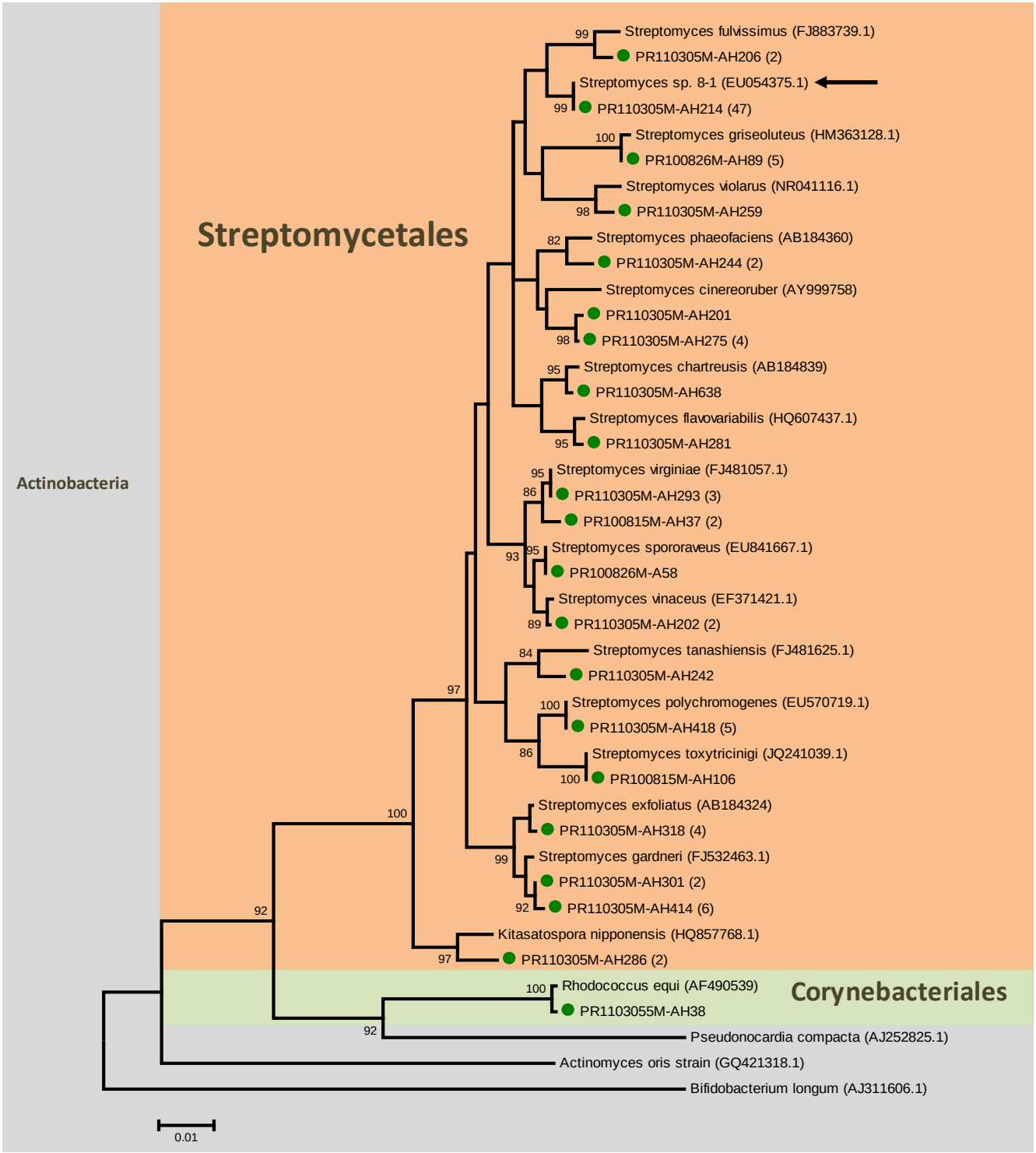

Figure 3.6: A 16S rDNA phylogenetic Neighbor-Joining (NJ) consensus tree of Actinobacteria isolated from *Cyphomyrmex minutus* exoskeleton (green circles). The number of isolates represented by selected sequences in the tree is shown in parentheses after the name. The phylogeny is based on partial 16S rDNA sequences of approximately 1470bp. Type strain and additional sequences were selected from Ribosomal Data Base and GenBank. The scale bar corresponds to 0.01 substitutions per site and bootstrap support values are ≥70% after 5,000 pseudoreplicates.

In the yeast cultivar isolates, the Corynebacteriales are represented by *Nocardia*, *Tsukamurella* and *Rhodococcus*. The latter was the only genus found associated with the ant exoskeleton. The Streptomycetales are represented by *Streptomyces*, the most common genus and by *Kitasatospora*. As before, the most frequent isolate was also *Streptomyces* sp. 8-1 EU054375 with 21 sequences (Figure 3.7, black arrow).

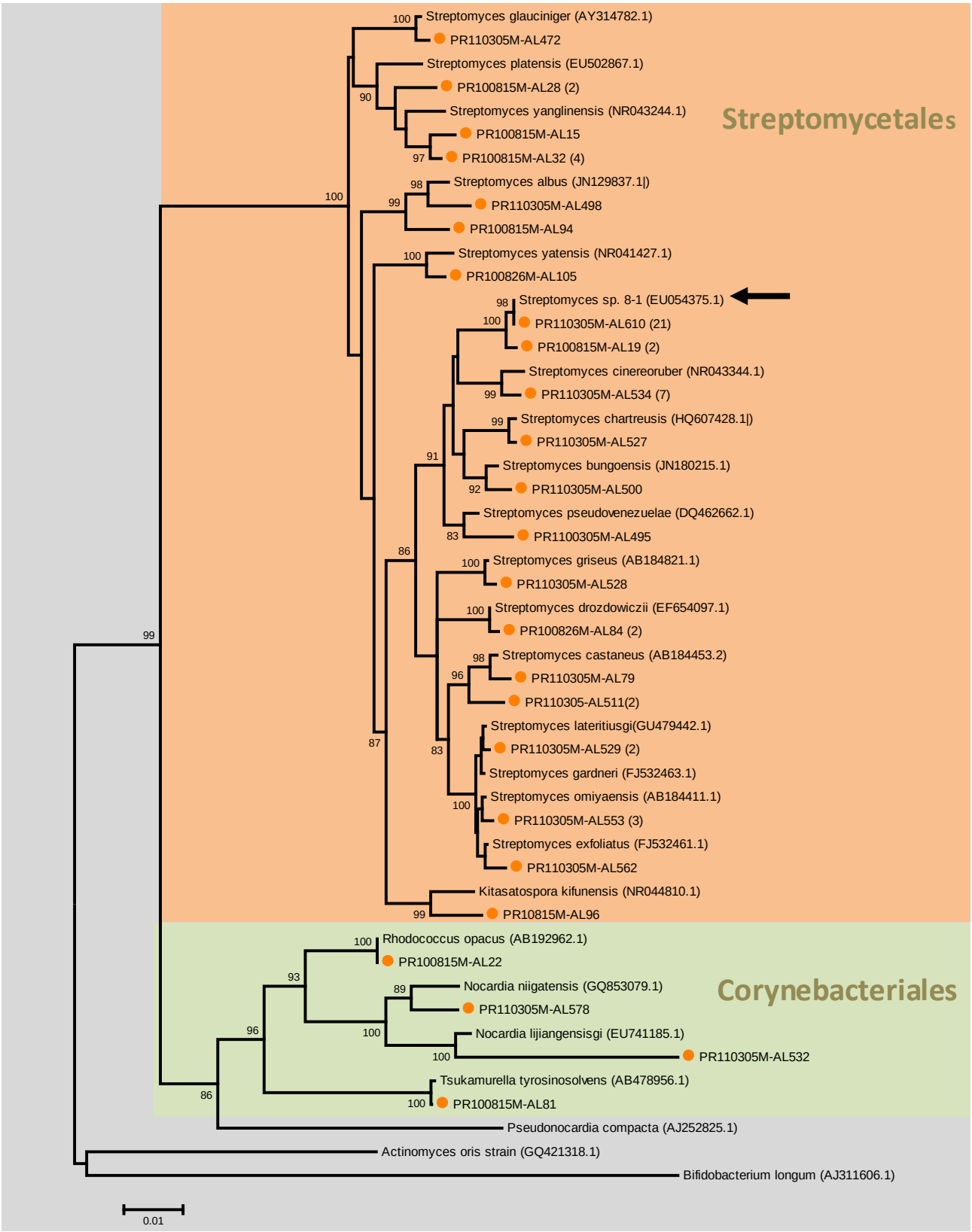
Streptomycetales
Corynebacteriales
Streptomyces glauciniger (AY314782.1)
PR110305M-AL472
Streptomyces platensis (EU502867.1)
PR100815M-AL28 (2)
Streptomyces yanglinensis (NR043244.1)
PR100815M-AL15
PR100815M-AL32 (4)
Streptomyces albus (JN129837.1|)
PR110305M-AL498
PR100815M-AL94
Streptomyces yatensis (NR041427.1)
PR100826M-AL105
Streptomyces sp. 8-1 (EU054375.1)
PR110305M-AL610 (21)
PR100815M-AL19 (2)
Streptomyces cinereoruber (NR043344.1)
PR110305M-AL534 (7)
Streptomyces chartreusis (HQ607428.1|)
PR110305M-AL527
Streptomyces bungoensis (JN180215.1)
PR110305M-AL500
Streptomyces pseudovenezuelae (DQ462662.1)
PR1100305M-AL495
Streptomyces griseus (AB184821.1)
PR110305M-AL528
Streptomyces drozdowiczii (EF654097.1)
PR100826M-AL84 (2)
Streptomyces castaneus (AB184453.2)
PR110305M-AL79
PR110305-AL511(2)
Streptomyces lateritiusgi(GU479442.1)
PR110305M-AL529 (2)
Streptomyces gardneri (FJ532463.1)
Streptomyces omiyaensis (AB184411.1)
PR110305M-AL553 (3)
Streptomyces exfoliatus (FJ532461.1)
PR110305M-AL562
Kitasatospora kifunensis (NR044810.1)
PR10815M-AL96
Rhodococcus opacus (AB192962.1)
PR100815M-AL22
Nocardia niigatensis (GQ853079.1)
PR110305M-AL578
Nocardia lijiangensisgi (EU741185.1)
PR110305M-AL532
Tsukamurella tyrosinosolvens (AB478956.1)
PR100815M-AL81
Pseudonocardia compacta (AJ252825.1)
Actinomyces oris strain (GQ421318.1)
Bifidobacterium longum (AJ311606.1)
0.01

Figure 3.7: A 16S rDNA phylogenetic Neighbor-Joining (NJ) consensus tree of Actinobacteria isolated from *Cyphomyrmex minutus* yeast cultivar (orange circles). The number of isolates represented by selected sequences in the tree is shown in parentheses after the name. The phylogeny is based on partial 16S rDNA sequences of approximately 1470bp. Type strain and additional sequences were selected from Ribosomal Data Base and GenBank. The scale bar corresponds to 0.01 substitutions per site and bootstrap support values are ≥70% after 5,000 pseudoreplicates.

3.4. DISCUSSION AND CONCLUSIONS

The microbial community and their ecological dynamics in the yeast agriculture is an understudied aspect of the fungus-growing ant symbiotic system. We presented critical points about the interaction between the yeast agriculture ant *C. minutus*, its defense mechanism and the potential symbiosis with Actinobacteria.

First, *C. minutus* workers do not present visible Actinobacteria growth over the propleural plate or the exoskeleton. However using SEM we observed bacterial growth on the forelegs exoskeleton and an unidentified coverage over the propleural plates (Figure 3.1). *Cyphomyrmex minutus* presents propleural plates with crypts covered with an unidentified substance. The crypts resembled previous morphological descriptions in other attine ant species and thus, have the potential to harbor Actinobacteria in them. However, minor morphological differences of the crypts related to their form and arrangement in the propleural plate were recognized when compared to other *Cyphomyrmex* species that are not part of the *rimosus* group (Currie et al. 2006). Further comparison with other members of the *rimosus* group is needed to explore these differences in detail.

The characterization of Actinobacteria communities associated with the ant exoskeleton and the yeast cultivar showed similar composition. Both communities shared *Streptomyces* as the most frequent isolate. Other common isolated genera included *Nocardia*, *Rhodococcus* and *Tsukamurella*. This is not surprising because attine ants manage or rearrange the cultivar yeast

pellets with their antennae, frontal legs, and mouth and propleural plates; hence coming in constant contact with other microorganisms in the community (Currie 2001a, Currie et al. 2006, Shultz and Brady 2008, Mehdiabadi and Shultz 2010). Based on our results, we interpret that there are almost no differences between the Actinobacteria communities associated with the ant exoskeleton and the yeast cultivar.

The most frequent isolated species of Actinobacteria was *Streptomyces* sp. 31 100% similar to *Streptomyces* sp. 8-1 (EU054375). This strain was isolated from 48% of the ant exoskeleton samples and from 33% of the yeast cultivar samples. High probability of isolating this particular strain from *C. minutus* exoskeleton and yeast cultivar indicates a potential role in the yeast agriculture symbiosis system. *Streptomyces* sp. 8-1 was isolated originally from "torrid zone forest soil" in China, but unfortunately no other information is available for the strain. The closest comparable type strain is *Streptomyces avermitilis*, an important industrial strain for the production of secondary metabolites (Omura et al. 2001). The predominant presence of *Streptomyces* strains in the yeast agriculture system suggests an initial acquisition from soil of secondary metabolite products with potential benefits for the ant and its cultivar. Furthermore, the low diversity in the ant and cultivar communities, as indicated by Simpson and Shannon indices, in combination with one highly prevalent member as suggested by Dominance and Eveness indicators, give support to the idea of a beneficial relationship. At least for *C. minutus* ants in Cambalache Forest *Streptomyces* sp. 31 seems to be prevalent enough to play a possible beneficial role in the community that may include defense mechanisms against pathogens by secondary metabolite production as described for other fungus-growing ant agricultural systems.

4. FUNGI ASSOCIATED WITH THE CULTIVAR AND THE REFUSE MATERIAL

4.1. INTRODUCTION

The ants in the monophyletic tribe Attini are characterized by their agricultural practices. In general the ants of this group collect organic material as substrate for their fungal cultivar (Currie 2001a). The cultivar is a Leucocoprinaceus fungus (Basidiomycota) that serves as the main source of food. The Attini ants and the cultivar have a coevolution history of over 50 million years (Shultz and Brady 2008). This interaction was described as an important example of symbiosis, an arms race between the ant and the cultivar to evolve and survive against pathogens (Currie 2001a). The ant-cultivar mutualism has evolved into 5 different agricultural practices: Lower Attini agriculture, Coral fungi agriculture, Yeast agriculture (*Cyphomyrmex rimosus* group), Higher domesticated agriculture and Leaf-cutter agriculture (Mehdiabadi 2010, Shultz and Brady 2008). *Cyphomyrmex* species members of the *rimosus* group (39 species) are the only Attini ants that do not cultivate fungi as mycelium, but rather in yeast form (Weber 1972, Shultz and Brady 2008, Mehdiabadi and Shultz 2010).

The cultivar reproduces clonally in the presence of the ants and is transmitted vertically from one nest to the other by the new queen (Currie et al. 1999). Clonal reproduction and nest environment make the cultivar vulnerable to pathogens. To protect the cultivar the ant developed complex hygienic behaviors that include: antennal inspection, nest rearrangement, cultivar and exoskeleton weeding and grooming, antimicrobial glandular secretion and association with antibiotic producing Actinobacteria among others (Murakami and Higashi 2007, De Finelinch and Boomsma 2010, Mehdiabadi and Shultz 2010, Pagnocca et al. 2012)

Escovopsis (Ascomycota) is the specialized parasite of the fungus-growing ant cultivar, but other microfungi and yeasts have been consistently isolated from Attini nests (Weber 1972,

Rodrigues et al. 2008). *Escovopsis* is an anamorphic and necrotic mycoparasite that belongs to the order Hypocreales of the Ascomycota (Reynolds and Currie 2004, Currie 2003). The transmission of the pathogen between nests is unknown, but it has been suggested to be horizontal because *Escovopsis* has not been reported for any other environment other than Attini ant nests (Currie 1999b, Bo et al. 2001, Reynolds and Currie 2004). In addition, *Escovopsis* is specific to the cultivar in four of the Attini agricultural practices infecting on average 39.7% of the studied cultivar gardens (Currie 2001b, Mehdiabadi and Shultz 2010). Infection rate varies between species and sampling sites from 11-75% (Currie 2001b, Rodrigues et al. 2008). *Escovopsis* infected colonies of *Atta* have smaller cultivar gardens and lower production of workers (Currie 2001b). On the other hand, the new queen does not carry pathogen inoculums, the infection does not start immediately after nest establishment and one *Escovopsis* species can be related to many Attini genera and vise versa (Currie et al. 1999, Currie 2001a, Seal et al. 2007, Pagnocca 2008). How the pathogen is transmitted has not been determined yet, but the possibility exists that other insects that live in the nest can serve as vectors for *Escovopsis* (Currie et al. 1999).

After inspecting the cultivar, substrate, nest material and other workers with the antennae, the ants start grooming and weeding refuse material. The ants lick and moisten the material with their mandibles and salivary secretions. The refuse material is disposed in dumps (Weber 1958, Bot et al. 2001, Seal et al. 2006). This adaptation appears to be similar for all the Attini and it is suggested as a standard behavior for all the agricultural practices (Weber 1958). The Attini ants that practice yeast agriculture are the smallest of all genera. Their nests consist of only one chamber, where the ants place the cultivar, the brood and the refuse material separately by areas (Weber 1958, Seal et al. 2006). In other Attini groups the ants have

multiple chambers and ants with more specialized behaviors (Weber 1958, Weber 1972, Currie 2001a, Bot et al. 2001).

Besides multiple chemical and mechanical defenses, the Attini nest is not a sterile environment. In addition to the cultivar, cultivar pathogen and the Actinobacteria symbionts, other microorganisms can colonize this environment (Weber 1972, Rodrigues et al. 2005b). In laboratory nests of *Atta cephalotes* a change in the mycoflora associated with the cultivar as a consequence of the plant substrate provided has been observed (Pagnocca et al. 2012). This suggests that the organic materials that ants bring into the nest might function as vectors for the mycoflora. Common soil fungi such as *Fusarium, Rhizopus* and *Trichoderma* and yeast in the genera *Cryptococcus, Pichia, Rhodotorula, Sporobolomyces* and *Trichosporon* were detected in environmental samples of *Atta* (Carreiro et al. 2002, Pagnocca et al. 2009, Pagnocca 2012).

Studies with multiple species of *Acromyrmex* showed a high prevalence of *Fusarium oxysporum* and *Cunninghamella binarae* in addition to 13 other genera (*Xylaria, Volutella, Penicillium, Paecilomyces, Monliella, Lecythophora, Thrichoderma, Cladosporium, Chaetomium, Eupenicillium, Aspergillus, Syncephalastrum* and *Mucor*) representing 10% of all isolates (Rodrigues et al. 2005a, Rodrigues et al. 2008). The above mentioned genera are common soil fungi and some of them are potential pathogens; although none of them appear to cause significant damage to the cultivar or to be as highly specialized as *Escovopsis* (Rodrigues et al. 2008). In *Acromyrmex* species, *F. oxysporum* and *C. binarae* do not seem to affect the cultivar garden. They appear to compete for nutrients in the same environment, acting as antagonists, but not as pathogens (Rodrigues et al. 2008).

In yeast agriculture, *Escovopsis* has not been isolated in association with the cultivar or any other part of the symbiosis. Additionally, there are no studies about the mycoflora associated with this community. The information about *Escovopsis* and other fungi in the Attini refuse material is limited. In *Atta colombica*, *Ecovopsis* is present in 48% of the sampled nests, while it was isolated from 66% of the refuse material sampled (Currie et al. 2001b). In this case, as expected, the prevalence of the pathogen is higher in the ants refuse material than in the cultivar. Proportions might vary between species and sampling sites, but have not yet been reported. Experimental work with *Acromyrmex* laboratory colonies showed that ant workers kept near refuse material dumps died sooner than non-exposed ants (Bot et al. 2001). The refuse material might represent another adaptation of the Attini ants to protect their main source of food against potential pathogens.

For many years, yeast agriculture was considered as the most primitive agricultural practice among Attini ants (Weber 1958, 1972, Mueller 2001). The *rimosus* group was described as the smallest ant species with the simplest nest and cultivar gardens among the Attini species. One important reason was that they cultivate a unicellular phase of fungi instead of a more complex multicellular form. Furthermore, they only add raw material as a substrate to their gardens such as insect corpses and feces (Weber 1952, Weber 1972, Brady and Shultz 2008). In addition, their cultivar can be found as free-living fungi in the environment (Mikheyev et al. 2010).

Molecular studies showed that the *rimosus* group is an intermediate group between the Lower and Higher Attini (Shultz and Brady 2008). The cultivar presents pleomorphisim and grows, in the ant presence, as yeast pellets created with salivary secretions and the provided substrate (Weber 1958, 1972). A recent study demonstrated that the primary nutrient source for the

cultivar is the regurgitated liquid nectar (De Finelincht and Boomsma 2010). Furthermore, the Leucocoprinae *C. minutus* cultivar itself has the ability to produce antifungal diketopipperazines (Wang et al. 1998). We wondered if behind the possible absence of the pathogen is a very successful adaptation between the *rimosus* group and its cultivar perhaps including other associated microorganisms.

The main goal of this study is to describe, for the first time, the microfungal community associated with the nest of the yeast agriculture ant *C. minutus* in Puerto Rico. We described the microfungi in association with the cultivar and the refuse material of the nest. Even though it is generally accepted that *Escovopsis* is not present in yeast agriculture gardens there are no studies reporting the presence or absence of the pathogen or any other microfungi associated with yeast agriculture.

4.2. Materials and Methods

4.2.1. Samples selection

We collected samples in two periods, 2010 rainy season and 2011 dry season at Cambalache Tropical Forest. Samples from stabilized artificial *C. minutus* nests were processed in the laboratory the same day of sampling. We selected 3 cultivar pellets from each nest. Separately, we selected 3 refuse material clusters from 6 different nests and prepare them for direct DNA isolation.

4.2.2. Microorganism at the cultivar and refuse material surface

We selected three different yeast cultivar clusters and 3 different refuse material samples to be fixed with 2.5% gluteraldehyde for 24 hour at 4°C in a 1.5mL microcentrifuge tubes. We washed the samples three times with phosphate buffer [0.1 M]. Samples were dehydrated

using serial ethanol washes (10%-100%) for 15 minutes each. Every time we changed liquids we centrifuged the samples for 30 seconds at 300 rpm. Dehydration was completed by critical point drying for 30 minutes. Dried samples were covered with gold/palladium to allow electron conductivity. Using the scanning electron microscope (SEM) we observed the yeast clusters (De Nollin and Borgers 1975, Gabriel 1982).

4.2.3. ISOLATION AND IDENTIFICATION OF THE MICROFUNGI ASSOCIATED FORM THE YEAST CULTIVAR

Cultivar pellets were washed, macerated and mixed with vortex in 1.5mL microcentrifuge tubes with 900µL of 0.7% NaCl. In triplicate, we inoculated 300 µL of each wash in Potato Dextrose Agar (PDA) (39 g Potato Dextrose Agar powder in a final volume of 1000mL of dH_2O). To avoid bacterial growth we supplemented the media with Penicillin and Streptomycin (0.05g/L). We spread the solution and incubated at 25°C. We checked the plates for growth every 24 hours. Selection and further purification was performed every day during four weeks. Multiple transfers were needed to obtain pure cultures. We classified isolates from each nest and identified macroscopic morphological characteristics.

4.2.4. DNA EXTRACTION AND ITS1/ITS2 AMPLIFICATION

We extracted total DNA from all isolates in pure culture with the Cetyl-trimethyl ammonium bromide (CTAB) modified protocol (Mueller et al. 1998, Vo et al. 2009). We macerated the cells in a frozen mortar (-80°C) until we obtained a fungal powder. The powder was transferred to 1.5 mL tubes with CTAB. To continue cell wall disruption, we subjected the samples to three cycles of freeze (-80°C) thawing (65°C). Later we treated the samples with chloroform followed by isopropanol precipitation and 100% ethanol washes. Samples were dried and then resuspended with TE 1:10 buffer and preserved at -20°C.

Approximately 40ng of DNA template were used for amplification of the ITS1/ITS2 in 50µL reactions, which included: 0.8x PCR buffer, 2.5nm $MgCL_{2,}$ 0.6µ M of each primer 0.16mM dNTps and 5U Taq polymerase per reaction. We used the following thermal parameters: 95°C 3´, 95°C 45´´, 52°C 45´´, 72°C 1´3´´and 72°C for 30 cycles. Fungal primers that amplify the ITS1/ITS2 region: ITS4 Reverse (5'TCCTCCGCTTATTGATATGC) and IT5 Forward (5'GGAAGTAAAAGTCGTAACAAGG) (White 1990) were used. We used 10ng/µL DNA amplification product that varies between 550-700 bp to sequence at the High-Throughput Genomics Unit of the University of Washington, Seattle, WA.

4.2.5. FUNGI FROM THE REFUSE MATERIAL

Each refuse material sample was processed separately. First we performed a DNA extraction with Cetyl-trimethyl ammonium bromide (CTAB) modified protocol (Mueller et al. 1998, Vo et al. 2009). We macerated refuse material clusters with a pestle in 1.5 mL tubes with CTAB. To disrupt cells we changed temperature from 65°C to -80°C for 10 minutes each time and repeated the process 3 times. Later we treated the samples with chloroform followed by isopropanol precipitation and 100% ethanol washes. Samples were dried and then resuspended with TE 1:10 buffer and stored at -20°C. We used a Gel/PCR DNA Fragment extraction kit (IBI Scientific) after Polymerase Chain Reaction of the ITS1 and ITS2. Approximately 40ng of purified DNA template was used for amplification in 50µL reactions that included: 0.8x PCR buffer, 2.5nM $MgCL_{2,}$ 0.6µM of each primer, 0.16mM dNTPs and 0.15 µL Taq polymerase per reaction. Fungal specific primers for PCR were used: ITS4 Reverse (5' -TCC TCC GCT TAT TGA ATG C-3´) and IT5 Forward (5'-GGA AGT AAA AGT CGT AAC AAG G-3´) (White 1990). PCR parameters used: 95°C 3´, 95°C 45´´, 51. °C

45´´, 72°C 1´3´´ 72°C during 30 cycles. Cloning was performed using pGEM-T and pGEM-T Easy vector System and the manufacturer recommended competent cells (Promega Corporation). Positive clones were selected by colony PCR using the vector primers SP6 (5´-TAC GAT TTA GGT GAC ACT ATA G-3´) and T7 (5´-TAA TAC GAC TCA CTA TAG GG-3´). PCR parameters used: 95°C 3´, 95°C 45´´, 45 °C 45´´, 72°C 1´3´´ 72°C during 30 cycles. Sequencing was performed with the vector primer SP6 at the High-Throughput Genomics Unit of the University of Washington, Seattle, WA. We used 10ng/µL DNA amplification product for the reaction.

4.2.6. DATA ANALYSIS

Sequences were edited and analyzed using Sequencher 3.0 (Gene Codes, Ann Arbor, MI) and Mega 5 (Tamura et al. 2011) programs. GenBank searches with BLASTn were performed to identify the fungi at the refuse material and the cultivar pellets. We determined fungal frequency and diversity (Shannon and Simpson indices) present in both samples.

4.3. RESULTS

4.3.1. EXPLORING THE CULTIVAR AND THE REFUSE MATERIAL SURFACE

Using SEM we studied the surface of the cultivar (Figure 4.1 A and B). We observed the yeast pellet as a whole and disrupted by zones. The intact surface of the pellet presented an unidentified substance covering all the yeast cells (Figure 4.1B). In figure 4.1A, we observed disrupted areas with pseudohyphal growth. The refuse material presented a very diverse community of microorganisms on its surface (Figure 4.1C-D). We identified bacterial and mycelial growth around possible plant residues.

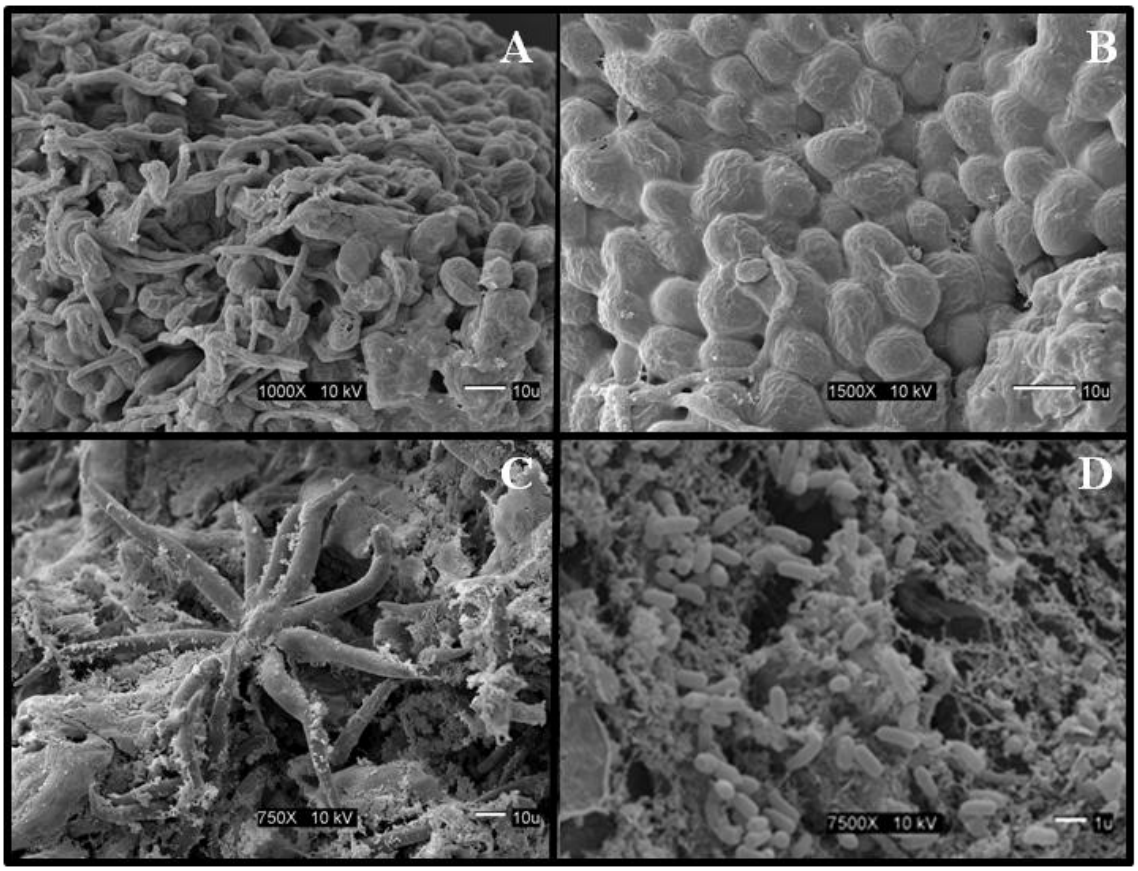

Figure 4.1: SEM microphotographs of cultivar and refuse material surfaces. (A) The cultivar pellet presents pleomorphisim growth with yeast and filamentous forms. (B) The yeast cells present an unidentified coverage. (C) Plant material surrounded by microorganism growth in the refuse material surface. (D) Bacilli growing over the refuse material surface.

4.3.2. FUNGI IDENTIFIED FROM THE CULTIVAR AND THE REFUSE MATERIAL OF *CYPHOMYRMEX MINUTUS*

From 26 different nests 156 isolates were obtained and 32 different genera in association with *C. minutus* cultivar were identified (Figure 4.2). Basidiomycota, Ascomycota and Zygomycota members were found associated with the cultivar (Figure 4.4). The most frequent genera were *Penicillium* (24%), *Aspergillus* (22%), *Fusarium* (9%), *Trichoderma* (8%), *Neurospora* (6%) and *Microdochium/Monographella* (5%). The rest of the isolates together represent 25% of the sample, less than 2% per genus.

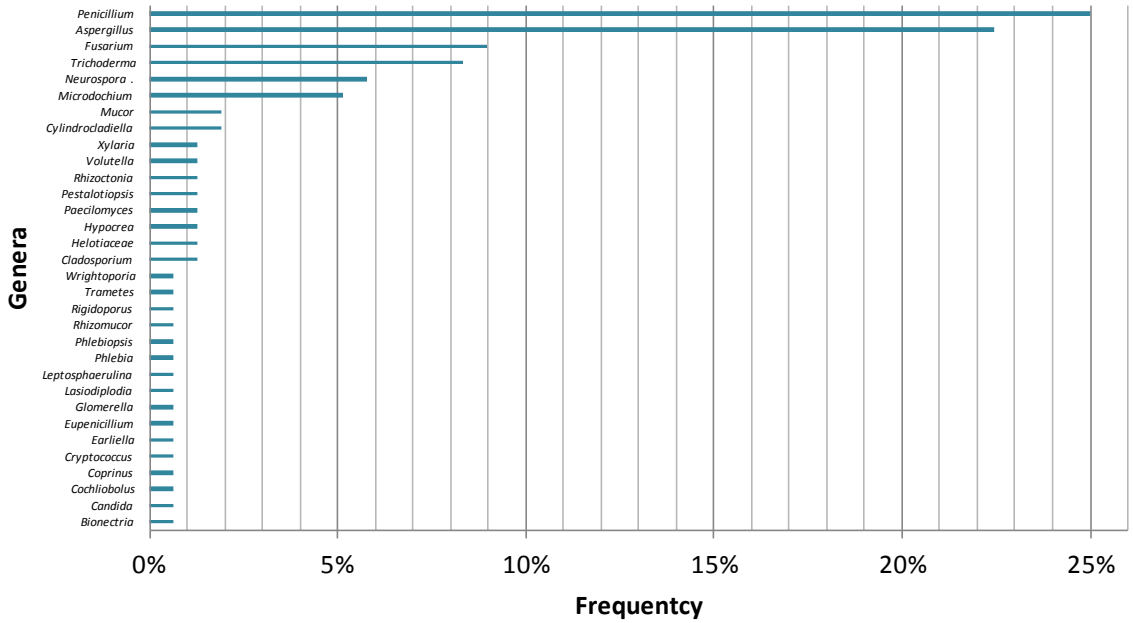

Figure 4.2: Frequency of microfungi cultures isolated from *C. minutus* cultivar.

We sequenced 145 different clones from refuse material samples. We identified 25 genera in the Basidiomycota and Ascomycota. The high frequency genera (Figure 4.3) in our culture-independent samples were *Microdochium/Monographella* (50%), *Fusidium* (8%), *Petriella* (7%) and *Leptosphaeria* (6%). The rest of the samples combined represent less than 29% of all studied clones.

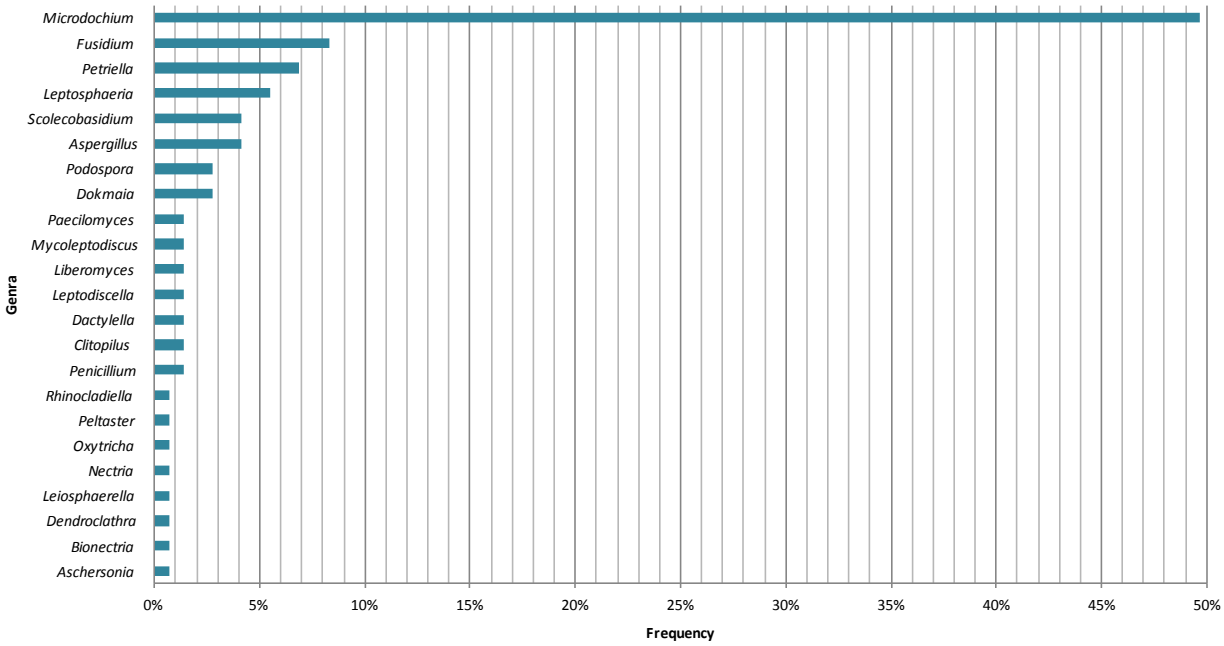

Figure 4.3: Frequency of clones identified from the refuse material samples by genus.

We consistently recovered from the cultivar and the refuse material the following genera: *Aspergillus, Bionectria, Microdochium/Monographella, Paecilomyces* and *Penicillium/Talaromyces* (Figures 4.2 and 4.3). In both samples we used ITS1/ITS2 as marker to identified organisms, but only analyzed them to genus level. We are not confident in species level identifications with this marker. In addition, many of the clones might represent the same fungus reported here as two different names (i.e. telomorph/anamorph).

4.3.3. DIVERSITY INDICES

Biodiversity of cultivable microfungi isolated from *C. minutus* cultivar was described using Simpson (S) and Shannon (H) indices (Table 4.1). The Simpson index evaluates the quantity of species in the sample and the richness in each sample (entropy). With the Dominance (D) calculation we can assess the presence of dominant species over the rest of the population.

The cultivable microfungal community associated with *C. minutus* cultivar (32 different genera in 156 samples) presented a diversity entropy indicator $S_{cultivar} = 0.0718$ and dominance value $D_{cultivar} = 0.9282$. In the fungal community associated with the refuse material we identified 25 genera from 145 clones. Using the same indices, we obtained $S_{refuse} = 0.1274$ for entropy and $D_{refuse} = 0.8726$ for dominance. The fungal community from the cultivar appears to be less entropic in terms of diversity than the community from the refuse material ($S_{cultivar} = 0.0718 < S_{refuse} = 0.1274$). The possibility of dominant species in the community is higher in the case of the community isolated from the cultivar ($D_{refuse} = 0.8726 < D_{cultivar} = 0.9282$).

In addition, we analyzed the same results using the Shannon index (H). This index evaluates the number of observed individuals for each species. In the case of the cultivable microfungal community from the cultivar we obtained $H_{cultivar} = 2.569$ index value and $H_{refuse} = 2.092$ for fungal clones from the refuse material. Values over 2.0 indicated variety of species in the community and high diversity. When we compared these two communities, the cultivar community appears to be slightly more diverse than the refuse material community ($H_{cultivar} = 2.569 < H_{refuse} = 2.092$). Eveness (E) indicates how close in species numbers are the communities. Eveness indicators for both communities exceed 1, indicating that the proportion of members of the each species is very similar in both communities (Table 4.1).

Table 4.1 Diversity indices estimated for fungal communities from the cultivar and the refuse material

Diversity index	Fungi identified from	
	Yeast cultivar	**Refuse material**
Simpson Index (S) S=0 low entropy (one species or few) S=1 high entropy (diverse community)	**0.0718**	**0.1274**
Dominance (D=1-S) D=0 all species are equally present D=1 one species dominates the community completely	**0.9282**	**0.8726**
Shannon index (H) H≤0 only one species at the community H>0 more than one species at the community	**2.569**	**2.092**
Eveness (E) E=1 similar proportion of all species in the community E>1 dissimilar proportion of the species in the community, dominant species	**1.692**	**1.496**

4.3.4. PHYLOGENETIC RELATIONSHIP BETWEEN THE IDENTIFIED FUNGI

Phylogenetic relationships between isolates from the cultivar were analyzed through Neighbor-Joining with p-distance and bootstrap support values after 5,000 pseudoreplicates. We included the closest previously identified sequences form GenBank using BLASTn and our isolates obtained in this study (Figure 4.4). We identified members of the Basidiomycota (green area), Ascomycota (orange area) and Zygomycota (purple area) (Figure 4.4). Ascomycota members were the most frequent including *Penicillium/Talaromyces*, *Aspergillus*, *Trichoderma*, *Neurospora* and *Microdochium*/*Mographella* (Figure 4.4).

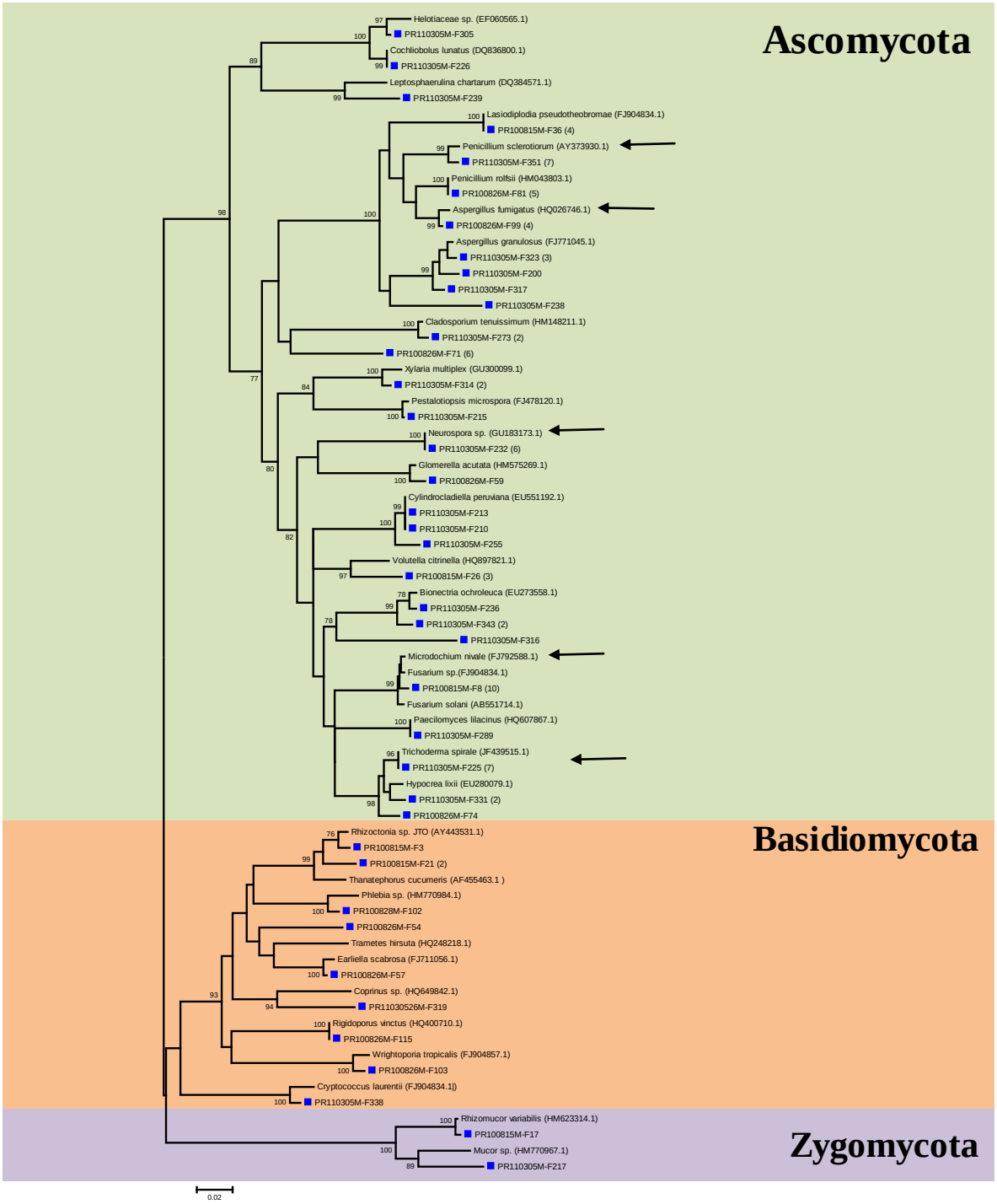

Figure 4.4: Neighbor-Joining tree of microfungi associated with *C. minutus* cultivar. Sequences from this study (blue squares) were compared with sequences from GenBank database using BLASTn. The tree was made using the information from the ITS region of the ribosomal DNA. The scale bar corresponds to 0.02 substitutions per site. Bootstrap support after 5,000 repetitions. The black arrows present the most frequent genera isolated.

Culture-independent identification of the fungi at the refuse material showed that only 1% of the genera belonged to Basidiomycota (Agaricales). The rest of the isolates were Ascomycota members in the following orders: Chaetothyriales, Eurotiales, Helotiales, Hypocreales, Magnaporthales, Pleosporales and Xylariales. The most frequent isolates were market with a black arrow (Figure 4.5). *Monographella,* anamorph of *Microdochium,* is the most prevalent followed by *Fusidium* (Hypocreales: Nectriaceae). It is important to highlight here that these samples were obtained by cloning and the majority of them belong to uncultivable fungi. Unfortunately, GenBank database does not have many closely related sequences to compare. Thus, we used the best sequence matches available to construct our trees.

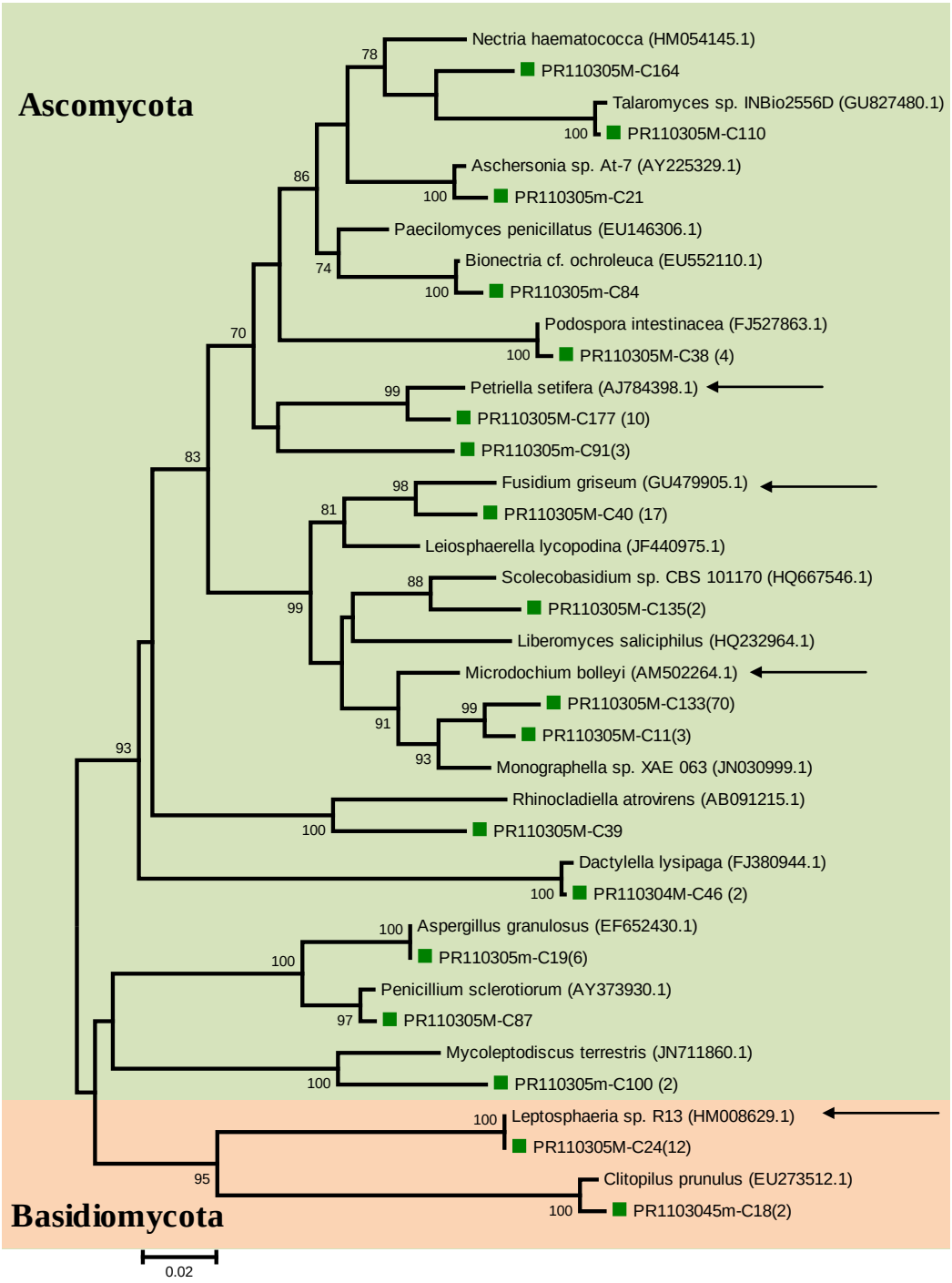

Figure 4.5: Neighbor-Joining tree of fungi associated with *C. minutus* refuse material. Sequences from this study (green squares) were compared with sequences from GenBank database using BLASTn. The tree was made using the information from the ITS region of the ribosomal DNA. The scale bar corresponds to 0.02 substitutions per site. Bootstrap support after 5,000 repetitions. The black arrows present the most frequent genera identified from the refuse material.

When comparing cultivar and the refuse material commnuties we observed five genera in common that were present in our samples: *Aspergillus, Bionectria, Microdochium, Penicillium* and *Talaromyces*. Interestingly, many of the recognized fungi are members of the order Hypocreales: *Aschersonia, Bionectria, Cylindrocladiella, Fusarium, Glomerella, Hypocrea, Paecilomyces, Nectria, Trichoderma* and *Volutella.* Among these Hypocreales *Bionectria* is the only one that was identified from both communities. *Escovopsis,* the specific fungus-growing ants cultivar pathogen, belongs in the Hypocreales.

Table 4.2: Comparison between identified fungi in association with the cultivar and the nest refuse material. Genera repeated in both environment are in red. Hypocreales members are identifiey with (*).

Classification	Cultivar	Refuse material
Basidiomycota	*Coprinus* sp.	*Clitopilus* sp.
	Cryptococcus sp.	
	Earliella sp.	
	Phlebia sp.	
	Rhizoctonia sp.	
	Rigidoporus sp.	
	Trametes sp.	
	Wrightoporia sp.	
Ascomycota	*Aspergillus* sp.	*Aschersonia**
	Bionectria sp.*	*Aspergillus sp*
	Candida sp	*Bionectria sp**
	Cladosporium sp	*Dactylella*
	Cochliobolus sp	*Dendroclathra sp*
	*Cylindrocladiella sp**	*Dokmaia sp*
	Eupenicillium sp	*Fusidium sp**
	*Fusarium spp **	*Leiosphaerella sp*
	*Glomerella sp**	*Leptodiscella sp*
	Helotiaceae sp	*Leptosphaeria sp*
	*Hypocrea sp**	*Liberomyces sp*
	Lasiodiplodia sp	*Microdochium sp/Monographella sp*
	Leptosphaerulina sp	*Mycoleptodiscus sp*
	Microdochium sp/Monographella sp	*Nectria sp**
	Neurospora sp.	*Paecilomyces sp*
	Paecilomyces sp.	*Peltaster sp*
	Penicillium sp/ Talaromyces sp	*Penicillium sp/ Talaromyces sp*
	Pestalotiopsis sp	*Petriella sp*
	Phlebiopsis sp	*Podospora sp*
	*Trichoderma sp**	*Rhinocladiella sp*
	*Volutella sp**	*Scolecobasidium sp*
	Xylaria sp	
Zygomycota	*Mucor sp*	
	Rhizomucor sp	

4.4. Discussion and Conclusions

The fungus-growing ant symbiosis system was described as an almost axenic environment for years. In the system, the ant cultivated a garden of basidiomycetous mycelial fungi (with the exception of *C. rimosus* group) (Weber 1955, Weber 1958). Later, the description of the system expanded as a complex environment consisting of various fungi that had specialized interactions including the pathogen *Escovopsis* (Currie 1999b). In addition, multiple mechanisms to protect the cultivar were explored including: (1) the mutualism with antibiotic producing bacteria, (2) ant hygienic behaviors (weeding, grooming, antennae activity, glandular and salivary secretions, etc) and (3) antimicrobial metabolites produced by other microorganisms present in the nest (bacteria, yeast or mycelial fungi different from the described symbionts) or the cultivar by itself (Wang et al. 1998, Currie et al. 1999a, Currie 2001a, Currie 2001b, Rodrigues et al. 2005a, Rodrigues et al. 2008). The interaction between the main symbionts in the system and other microorganisms remains understudied, although recent works suggest that additional defense mechanisms may be involved while in some cases parasitism or competence for the same environment may be occurring (Rodrigues et al. 2005b, Rodrigues et al. 2008, Pagnocca et al. 2008, Little and Currie 2009, Pagnocca et al. 2012).

Previous to this study there was no information about the actinobacteria community or the presence of *Escovopsis* in the yeast agriculture system. Our results established the absence of *Escovopsis*, the fungus-growing ant specialized pathogen, from the cultivar and refuse material of *C. minutus* in Cambalache Forest. We propose several alternative scenarios, which are not necessarily mutually exclusive. (1) *Escovopsis* is not present in Puerto Rico. In order to demonstrate this assumption we need to study the mycoflora associated with other species

of fungus-growing ants in Puerto Rico (*Trachymyrmex jamaicensis, Mycetophylax conformis* and *Mycocepurus smithii*), which do not practice yeast agriculture (Wheeler 1862). (2) The *rimosus* group prevents growth of pathogens through antimicrobial secretions that induce the cultivar pleomorphisim, but we do not know the mechanism. Based on nest observation, in addition to hygienic behavior, *C. rimosus* regurgitates liquid and insect feces as substrate for the cultivar, and then the ants apply the substrate to the pellets through licking (De Finelinch and Boomsma 2010). The regurgitated substrate might contain salivary and digestive secretions with antimicrobial capabilities. Regurgitation is unique to yeast agriculture ants (De Finelinch and Boomsma 2010). (3) The cultivar in yeast form cannot be infected by the fungus-growing cultivar pathogen. The yeast form decreases the exposed area available for infection; hence preventing *Escovopsis* from penetrating the mycelium and secreting necrotic enzymes, which initiate infection (Currie 2001b, Reynolds and Currie 2004). (4) Another possibility is that the cultivar in yeast form has the ability to protect itself. Leucoagaricus cultivar specialized to live in association with yeast agriculture ants evolved about 25 million years ago with the possibility of having its own defenses against pathogens (Mikheyev et al. 2010). Such defenses might have included the cultivar antifungal diketopipperazines or some other similar adaptation (Wang et al. 1998). (5) Finally, other microorganisms present in the system might compete for nutrients and resources not available to *Escovopsis* and/or have antagonistic relationships with pathogens not yet reported (Rodrigues 2008, Freinkman et al. 2009).

The fungal community living in association with the yeast agriculture ant *C. minutus* was studied in an attempt to identify potential fungal antagonists. If we compare the cultivar and the refuse material communities we observe that five genera were isolated from both the

cultivar and the refuse material: *Aspergillus, Penicillium/Talaromayces, Microdochium/Monographella, Bionectria and Paecilomyces* (Table 4.2*)*. All of them are Ascomycota, like *Ecovopsis*.

In terms of diversity, both communities show relatively low diversity according to the calculated indices with one or four possible dominant species over the rest of the community. In the case of the cultivar, *Penicillium* and *Aspergillus* appear to be dominant. As a consequence of their arial spores propagation system these two genera might have an advantage growing in laboratory medium over other species. However, these two genera have been isolated from other fungus-growing ant nests (Table 4.3). *Penicillium* and *Aspergillus* species were identified from *Trachymyrmex septentrionalis*, *Atta* spp., *Acromyrmex* spp., *Cyphomyrmex wheeleri* (Lower agriculture) nests (Weber 1955, Rodrigues et al. 2005a, Rodrigues et al. 2008, Rodrigues et al. 2011). Members of *Aspergillus* and *Penicillium* also have cellulose degradation capabilities that allow them to affect several plants and their fruits (Wood et al. 1989, de Vries and Visser 2001) and can be transported into the nest. In addition, some species of *Aspergillus*, such as *A. ochraceus,* are facultative entomopathogens (Lage et al. 2001)**.** Both genera might have the capability of infecting the cultivar, but because they are considered common soil saprofitic fungi and they were present in a low frequency this posibility is unlikely (Rodrigues et al. 2005a, Rodrigues et al. 2008, Rodrigues et al. 2011). Hence, *Aspergillus* and *Penicillium* are part of the normal soil and the nest mycoflora, which can sometimes overgrow in the ant nest (Steiman 1995,Currie 2001a, Rodrigues et al. 2008).

The diversity indices also indicate that between both communities the refuse material fungal community is more diverse. These results might be a consequence of the culture independent

used, which takes in consideration uncultivable fungi. The most dominant group in this community appears to be *Microdochium,* which was also isolated from the cultivar pellets in a lower frequency. In addition, this genus has never been reported in any other fungus-growing ant nests (Table 4.3). Members of *Microdochium* are well known plant pathogens (Ernest 2011). Recently, cyclosporine A was isolated from the estuarine species *M. nivale,* which shows antifungal activity against species of *Aspergillus, Trichophyton, Microsporium* and *Fusarium* (Bhosale et al. 2011). As suggested by Rodrigues et al. (2011), microfungi present in the Attini nest with antifungal capabilities may indicate an additional protection from pathogenic organisms.

Escovopsis belongs in the order Hypocreales. We identified 9 members of this order from both communities (*Aschersonia, Bionectria, Cylindrocladiella, Fusarium, Glomerella, Hypocrea, Nectria, Trichoderma* and *Volutella*). The only Hypocreales genus in common to both communities was *Bionectria,* a common soil fungus (Wang 2011, Freinkman 2009), which has also been identified in association with wood-feeding bark beetle (Freinkman 2010). *Bionectria* spp. have been reported from the Attini ant *Apterostigma dentigerum* cultivar (Table 4.3) (Freinkman 2010). *Bionectria* species isolated from *A. dentigerum* nests produce Bionectriol A, a polyketide glycoside who's role in the interaction remains unknown. However, similar polypeptide glycosides produced by *Streptomyces* have significant activity against antibiotic resistant staphylococci and enterococci (Herold et al. 2005).

Paecilomyces species have been describe from decaying plants and different soil samples including high heat resistant strains isolated from food (Sampson et al. 2009). *Paecilomyces fumosoroseus* is an entomopathogen that affects the Russian wheat aphid, *Diuraphis noxia* (Mesquita et al. 2001), which also degrades cellulose and lining efficiently (Kapoor et al.

1978). Another entomopathogen species is *P. lilacinus*, which produces proteases and chitinases that can alter the eggshell structure of nematodes (Khan et al. 2004). Our results identified a closely related species (Figure 4.4) indicating that the nest environment is subjected to common soil entomopathogens.

Several genera that were identified from *C. minutus* in this study were also recorded from *C. wheeleri* (Rodrigues et al. 2011). A detailed comparison for both ants is shown in Table 4.3. Although *C. wheeleri* does not practice yeast agriculture, the two ants belong in the same genus and cultivate closely related *Leucoagaricus* fungi (Mikheyev et al. 2010). The two species share similar behavior characteristics such as nest establishment, architecture and colony size (Weber 1958, Weber 1972). *Phlebia* species (Basidiomycota) were isolated in low frequency for both ant species. *Phebia gigantea*, a well-known member of this genus, causes white rot on turf grasses, demonstrating cellulose and lignin degradation capabilities (Sartain and Volk 1983).

Other Ascomycota genera identified from both *Cyphomyrmex* species are: *Paecilomyces, Penicillium, Trichoderma, Fusarium, Eupenicillium, Cladosporium, Hypocrea, Leptosphaerulina* and *Podospora*. *Paecilomyces* and *Penicillium* were also isolated from other fungus-growing ants as mentioned above. *Fusarium* sp. and *Trichoderma* sp. appear to be frequent invaders of the fungus-growing ants without causing any negative effect in the community (Rodrigues et al. 2005a, 2008, 2011). Some authors consider them garden weeds that can be influenced by sampling season, ant species and geographical location (Currie 1999b, Rodrigues et al. 2005, 2011). In addition, *Candida* species associated with fungus-growing ants show mycotoxin activity that affects other fungi in the community (Pagnocca

2008). The yeast *Cryptococcus* also presents an inhibitory effect on the specific pathogen *Escovopsis* in *Atta texana* nests (Pagnocca 2008, Rodrigues et al. 2011). Both yeasts were isolated from *C. minutus* nests in Puerto Rico (Table 4.3).

In this study, we show the absence of *Escovopsis* in *C. minutus* nests and the presence of a fungal community comparable to other fungus-growing ant nests. Further studies are needed to understand and explore these fungi capabilities and roles in association with yeast-growing ants. We determine that genera like *Penicillium*, *Aspergillus*, *Fusarium* and *Trichoderma* are present in almost every sampled nest (Table 4.3). Nevertheless, these microfungi do not affect the cultivar or seem to act as pathogens. They appear to compete for the nutrients in the fungus-growing ant systems as they do in their natural environment (Rodrigues et al. 2009, 2011). Genera such as *Bionectria*, *Microdochium* and *Paecilomyces* produce antifungal compounds or can be potential entomopathogens. Rodrigues et al. (2009) suggest that microfungi in the fungus-growing ant communities can be controlling nutrient competition and as a consequence the ant and potential cultivar pathogens. Further studies must concentrate efforts in understanding *Candida*, *Cryptococcus*, *Bionectria*, *Microdochium* and *Paecilomyces* antibiosis and their potential role in yeast cultivar defense.

Table 4.3: Comparison between the fungi community identified from *Cyphomyrmex minutus* (cultivar and refuse material) and other fungus-growing ants (cultivar, refuse material or ant body). All the fungi listed were identified from *C. minutus* (*) in this study.

		Isoleted from		
Clasification	**Classification**	**Cultivar**	**Refuse material**	**Ant body**
Basidiomycota	*Clitopilus*		*Cyphomyrmex minutus**	
	Coprinus	*Cyphomyrmex minutus**		
	Cryptococcus	*Cyphomyrmex minutus**	*Myrmicocrypta* sp (Pagnocca et al. 2009)	*Atta* spp (Pagnocca et al. 2008)
		Atta texana (Rodrigues et al. 2009)		
	Earliella	*Cyphomyrmex minutus**		
	Phlebia	*Cyphomyrmex minutus**		
		Cyphomyrmex wheeleri (Rodrigues et al. 2011)		
	Rhizoctonia	*Cyphomyrmex minutus**		*Acromyrmex* spp (Van Borm et al. 2002)
		Atta texana (Rodrigues et al. 2011)		
	Rigidoporus	*Cyphomyrmex minutus**		
	Trametes	*Cyphomyrmex minutus**		
	Wrightoporia	*Cyphomyrmex minutus**		

Table 4.3: (Cont.)

Clasification	Classification	Isoleted from		
		Cultivar	**Refuse material**	**Ant body**
Ascomycota	*Arthrobotrys*	*Atta sexdens rubropilosa* (Rodrigues et al. 2005a)	*Cyphomyrmex minutus**	
	Aschersonia		*Cyphomyrmex minutus**	
	Aspergillus	*Cyphomyrmex minutus**	*Cyphomyrmex minutus**	*Atta laevigata* (Pagnocca et al. 2008)
		Atta sexdens rubropilosa (Rodrigues et al. 2005a)	*Atta sexdens rubropilosa* (Rodrigues et al. 2005a)	
		Trachymyrmex seotentrionalis (Weber 1955, Rodrigues et al. 2011)		
		Cyphomyrmex wheeleri (Rodrigues et al. 2011)		
		Acromyrmex spp (Rodrigues et al. 2008)		
		Atta texana (Rodrigues et al. 2011)		
	Bionectria	*Cyphomyrmex minutus**	*Cyphomyrmex minutus**	
		Apterostigma dentigerum (Freinkman et al. 2009)**		
	Candida	*Cyphomyrmex minutus**	*Myrmicocrypta* sp (Pagnocca et al. 2009)	*Atta* spp (Pagnocca et al. 2008)
		Atta texana (Rodrigues et al. 2009)		
	Cladosporium	*Cyphomyrmex minutus**	*Atta sexdens rubropilosa* (Rodrigues et al. 2005a)	*Atta* spp (Pagnocca et al. 2008)
		Atta sexdens rubropilosa (Rodrigues et al. 2005a)		
		Acromyrmex hispidus (Rodrigues et al. 2008)		
		Chyphomyrmex wheeleri (Rodrigues et al. 2011)		
	Cochliobolus	*Cyphomyrmex minutus**		
	Cylindrocladiella	*Cyphomyrmex minutus**		
	Dactylella		*Cyphomyrmex minutus**	

Table 4.3: (Cont.)

		Isoleted from		
Clasification	**Classification**	**Cultivar**	**Refuse material**	**Ant body**
Ascomycota	*Dendroclathra*		*Cyphomyrmex minutus**	
	Eupenicillium	*Cyphomyrmex minutus**		
		Acromyrmex hispidus (Rodrigues et al. 2008)		
		Chyphomyrmex wheeleri (Rodrigues et al. 2011)		
		Trachymyrmex septentrionalis (Rodrigues et al. 2011)		
		Atta texana (Rodrigues et al. 2011)		
	Fusarium	*Cyphomyrmex minutus**	*Atta sexdens rubropilosa* (Rodrigues et al. 2005a)	*Atta* spp (Pagnocca et al. 2008)
		Acromyrmex spp (Rodrigues et al. 2008)		
		Atta sexdens rubropilosa (Rodrigues et al. 2005a)		
		Chyphomyrmex wheeleri (Rodrigues et al. 2011)		
		Trachymyrmex septentrionalis (Rodrigues er al 2011)		
	Fusidium		*Cyphomyrmex minutus**	
	Glomerella	*Cyphomyrmex minutus**		
	Helotiaceae	*Cyphomyrmex minutus**		
	Hypocrea	*Cyphomyrmex minutus**		
		Cyphomyrmex wheeleri (Rodrigues et al. 2011)		
	Lasiodiplodia	*Cyphomyrmex minutus**		
		Atta texana (Rodrigues et al. 2011)		

Table 4.3: (Cont.)

		Isoleted from		
Clasification	**Classification**	**Cultivar**	**Refuse material**	**Ant body**
Ascomycota	*Leiosphaerella*		*Cyphomyrmex minutus**	
	Leptodiscella		*Cyphomyrmex minutus**	
	Leptosphaeria	Cyphomyrmex wheeleri (Rodrigues et al. 2011)	*Cyphomyrmex minutus**	
	Leptosphaerulina	*Cyphomyrmex minutus**		
		Cyphomyrmex wheeleri (Rodrigues et al. 2011)		
		Atta texana (Rodrigues et al. 2011)		
	Liberomyces		*Cyphomyrmex minutus**	
	Microdochium	*Cyphomyrmex minutus**	*Cyphomyrmex minutus**	
	Mycoleptodiscus		*Cyphomyrmex minutus**	
	Nectria	*Trachymyrmex septentrionalis* (Rodrigues et al. 2011)	*Cyphomyrmex minutus**	
	Neurospora .	*Cyphomyrmex minutus**		
	Paecilomyces	*Cyphomyrmex minutus**	*Cyphomyrmex minutus**	
		Acromyrmex coronatus (Rodrigues et al. 2008)		
		Trachymyrmex septentrionalis (Rodrigues et al. 2011)		
		Cyphomyrmex wheeleri (Rodrigues et al. 2011)		
		Atta texana (Rodrigues et al. 2011)		

Table 4.3: (Cont.)

		Isoleted from		
Clasification	**Classification**	**Cultivar**	**Refuse material**	**Ant body**
Ascomycota	*Penicillium*	*Cyphomyrmex minutus**	*Cyphomyrmex minutus**	*Atta spp* (Pagnocca et al. 2008)
		Atta sexdens rubropilosa (Rodrigues et al. 2005a)	*Atta sexdens rubropilosa* (Rodrigues et al. 2005a)	
		Acromyrmex spp (Rodrigues et al 2008)		
		Trachymyrmex septentrionalis (Rodrigues et al. 2011, Weber 1955)		
		Cyphomyrmex wheeleri (Rodrigues et al 2011)		
		Atta texana (Rodrigues et al 2011)		
		Cyphomyrmex wheeleri (Rodrigues et al 2011)		
	Pestalotiopsis	*Cyphomyrmex minutus**		
		Atta texana (Rodrigues et al 2011)		
	Petriella		*Cyphomyrmex minutus**	
	Phlebiopsis	*Cyphomyrmex minutus**		
	Podospora	*Cyphomyrmex wheeleri* (Rodrigues et al 2011)	*Cyphomyrmex minutus**	
	Rhinocladiella		*Cyphomyrmex minutus**	
	Scolecobasidium		*Cyphomyrmex minutus**	

Table 4.3: (Cont.)

		Isoleted from		
Clasification	**Classification**	**Cultivar**	**Refuse material**	**Ant body**
Ascomycota	*Trichoderma*	*Cyphomyrmex minutus**	*Atta sexdens rubropilosa* (Rodrigues 2005a)	*Atta* spp (Pagnocca et al 2008)
		Acromyrmex spp (Rodrigues et al 2008)		
		Atta sexdens rubropilosa (Rodrigues 2005a)		
		Trachymyrmex septentrionalis (Rodrigues er al 2011)		
		Atta texana (Rodrigues et al 2011)		
		Cyphomyrmex wheeleri (Rodrigues et al 2011)		
	Volutella	*Cyphomyrmex minutus**		
		Acromyrmex spp (Rodrigues et al 2008)		
	Xylaria	*Cyphomyrmex minutus**		
		Acromyrmex spp (Rodrigues et al 2008)		
		Atta texana (Rodrigues et al 2011)		
		Trachymyrmex septentrionalis (Rodrigues er al 2011)		
Zygomycota	*Mucor*	*Cyphomyrmex minutus**		
		Trachymyrmex septentrionalis (Weber 1955, Rodrigues et al 2011)		
		Cyphomyrmex wheeleri (Rodrigues et al 2011)		
		Acromyrmex laticeps (Rodrigues et al 2008)		
	Rhizomucor	*Cyphomyrmex minutus**		
		Bionectria was isolated from *Apterostigma dentigerum* cultivar substrate (Freinkman et al 2009)**		

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Appendix A: Actinobacteria isolated from Cyphomyrmex minutus exoskeleton and cultivar during the 2 sampling times (Rainy and Dry Season)

Isolates	General information				Similar to				
	Identifiction code		Sampling	Isolated from	Accession	Description	Max score	Total score	Query coverage
Kitasatospora sp 3	PR110305m-	279	Dry	Ant exoeskeleton	HQ857768.1	*Kitasatospora nipponensis* strain H2-4	2385	2385	94%
Kitasatospora sp 3	PR110305m-	286	Dry	Ant exoeskeleton	HQ857768.1	*Kitasatospora nipponensis* strain H2-4	2390	2390	95%
Rhodococcus sp1	PR100815m-	38	Rainy	Ant exoeskeleton	DQ150573.1	*Rhodococcus equi* strain ATCC 33703	1354	1354	100%
Streptomyces sp 1	PR100826m-	64	Rainy	Ant exoeskeleton	AB184178.1	*Streptomyces aburaviensis* NBRC 12830	2475	2475	100%
Streptomyces sp 11	PR110305m-	205	Dry	Ant exoeskeleton	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173195	1430	1430	100%
Streptomyces sp 11	PR110305m-	246	Dry	Ant exoeskeleton	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173196	2481	2481	96%
Streptomyces sp 11	PR110305m-	318	Dry	Ant exoeskeleton	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173197	2523	2523	97%
Streptomyces sp 11	PR110305m-	319	Dry	Ant exoeskeleton	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173198	1454	1454	100%
Streptomyces sp 11	PR110305m-	476	Dry	Ant exoeskeleton	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173199	2494	2494	97%
Streptomyces sp 11	PR110305m-	479a	Dry	Ant exoeskeleton	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173200	2512	2512	97%
Streptomyces sp 12	PR100815m-	37	Rainy	Ant exoeskeleton	HQ850408.1	*Streptomyces flaveus* strain S13 16S	2597	2597	100%
Streptomyces sp 12	PR110305m-	336	Dry	Ant exoeskeleton	HQ850408.1	*Streptomyces flaveus* strain S13 16S	2508	2508	97%
Streptomyces sp 13	PR110305m-	281	Dry	Ant exoeskeleton	HQ607437.1	*Streptomyces flavovariabilis* strain 1184	2431	2431	97%
Streptomyces sp 15	PR110305m-	206	Dry	Ant exoeskeleton	FJ883739.1	*Streptomyces fulvissimus* strain cfcc3058	2355	2355	97%
Streptomyces sp 15	PR110305m-	624	Dry	Ant exoeskeleton	NR041210.1	*Streptomyces fulvissimus* strain NBRC 3717	2431	2431	97%
Streptomyces sp 16	PR110305m-	301	Dry	Ant exoeskeleton	FJ532463.1	*Streptomyces gardneri* strain HBUM175034	2477	2477	97%
Streptomyces sp 17	PR100826m-	49	Rainy	Ant exoeskeleton	HM363128.1	*Streptomyces griseoluteus* strain P510	2508	2508	96%
Streptomyces sp 17	PR100826m-	71	Rainy	Ant exoeskeleton	HM363128.1	*Streptomyces griseoluteus* strain P510	1308	1308	100%
Streptomyces sp 17	PR100826m-	83	Rainy	Ant exoeskeleton	HM363128.1	*Streptomyces griseoluteus* strain P510	2459	2459	96%
Streptomyces sp 17	PR100826m-	89	Rainy	Ant exoeskeleton	HM363128.1	*Streptomyces griseoluteus* strain P510	2510	2510	97%
Streptomyces sp 17	PR100826m-	91	Rainy	Ant exoeskeleton	HM363128.1	*Streptomyces griseoluteus* strain P510	2508	2508	96%
Streptomyces sp 17	PR100826m-	101	Rainy	Ant exoeskeleton	HM363128.1	*Streptomyces griseoluteus* strain P510	2479	2479	97%
Streptomyces sp 19	PR100815m-	34	Rainy	Ant exoeskeleton	HQ244456.1	*Streptomyces hygroscopicus* subsp. glebosus	1011	1011	99%
Streptomyces sp 19	PR100815m-	35	Rainy	Ant exoeskeleton	AJ781386.1	*Streptomyces hygroscopicus* subsp. glebosus	2617	2617	99%
Streptomyces sp 20	PR100826m-	55	Rainy	Ant exoeskeleton	NR043822.1	*Streptomyces kanamyceticus* strain NRRL B-2535	1360	1360	100%
Streptomyces sp 21	PR110305m-	255	Dry	Ant exoeskeleton	AB184531.1	*Streptomyces kasugaspinus* NBRC 13852	2274	2274	93%
Streptomyces sp 23	PR110305m-	228	Dry	Ant exoeskeleton	GU479442.1	*Streptomyces lateritius* strain A25	2473	2473	97%
Streptomyces sp 23	PR110305m-	298	Dry	Ant exoeskeleton	GU479442.1	*Streptomyces lateritius* strain A25	2460	2460	95%
Streptomyces sp 23	PR110305m-	405	Dry	Ant exoeskeleton	GU479442.1	*Streptomyces lateritius* strain A25	2523	2523	97%
Streptomyces sp 23	PR110305m-	414	Dry	Ant exoeskeleton	AF454764.1	*Streptomyces lateritius*	2497	2497	97%
Streptomyces sp 23	PR110305m-	622	Dry	Ant exoeskeleton	GU479442.1	*Streptomyces lateritius* strain A25	2464	2464	95%
Streptomyces sp 23	PR110305m-	643	Dry	Ant exoeskeleton	GU479442.1	*Streptomyces lateritius* strain A25	2518	2518	97%
Streptomyces sp 26	PR110305m-	244	Dry	Ant exoeskeleton	HQ607439.1	*Streptomyces phaeofaciens* strain 1187	2451	2451	95%
Streptomyces sp 26	PR110305m-	566	Dry	Ant exoeskeleton	HQ607439.1	*Streptomyces phaeofaciens* strain 1187	2440	2440	97%
Streptomyces sp 27	PR110305m-	212	Dry	Ant exoeskeleton	FJ486292.1	*Streptomyces platensis* strain HBUM82935	2588	2588	98%
Streptomyces sp 28	PR110305m-	249	Dry	Ant exoeskeleton	HQ844476.1	*Streptomyces polychromogenes* strain JSHS31	2459	2459	95%
Streptomyces sp 28	PR110305m-	253	Dry	Ant exoeskeleton	HQ844476.1	*Streptomyces polychromogenes* strain JSHS31	2451	2451	93%
Streptomyces sp 28	PR110305m-	270	Dry	Ant exoeskeleton	HQ844476.1	*Streptomyces polychromogenes* strain JSHS31	2447	2447	93%
Streptomyces sp 28	PR110305m-	413	Dry	Ant exoeskeleton	EU570719.1	*Streptomyces polychromogenes* strain 173069	2507	2507	97%
Streptomyces sp 28	PR110305m-	418	Dry	Ant exoeskeleton	EU570719.1	*Streptomyces polychromogenes* strain 173069	2521	2521	97%
Streptomyces sp 30	PR110305m-	211	Dry	Ant exoeskeleton	HQ238364.1	*Streptomyces sindenensis* strain XA66	1502	1502	98%
Streptomyces sp 31	PR100815m-	25	Rainy	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	2556	2556	99%
Streptomyces sp 31	PR100826m-	59	Rainy	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	1223	1223	100%
Streptomyces sp 31	PR100826m-	60	Rainy	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	1227	2412	97%
Streptomyces sp 31	PR100815m-	66	Rainy	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	1238	1238	100%
Streptomyces sp 31	PR110305m-	210	Dry	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	1489	1489	100%
Streptomyces sp 31	PR110305m-	214	Dry	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	2486	2486	95%

Appendix A: Continuation

Streptomyces sp 31	PR110305m-	630	Dry	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	2521	2521	97%
Streptomyces sp 31	PR110305m-	632	Dry	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	2497	2497	97%
Streptomyces sp 31	PR110305m-	646	Dry	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	2521	2521	98%
Streptomyces sp 31	PR100826m-	44	Rainy	Ant exoeskeleton	EU054375.1	*Streptomyces* sp. 8-1	917	917	99%
Streptomyces sp 32	PR100815m-	43	Rainy	Ant exoeskeleton	FN646652.1	*Streptomyces spororaveus* DLS-33	1230	1230	100%
Streptomyces sp 32	PR100826m-	58	Rainy	Ant exoeskeleton	EU841667.1	*Streptomyces spororaveus* strain HBUM173231	2567	2567	98%
Streptomyces sp 32	PR110305m-	256	Dry	Ant exoeskeleton	FN646652.1	*Streptomyces spororaveus* DLS-33	1445	1445	99%
Streptomyces sp 32	PR110305m-	257	Dry	Ant exoeskeleton	FN646652.1	*Streptomyces spororaveus* DLS-33	1483	1483	99%
Streptomyces sp 33	PR110305m-	242	Dry	Ant exoeskeleton	FJ481625.1	*Streptomyces tanashiensis* strain HBUM173179	2420	2420	95%
Streptomyces sp 34	PR100815m-	106	Rainy	Ant exoeskeleton	JQ241039.1	*Streptomyces toxytricini* strain HNU-EA27	2627	2627	100%
Streptomyces sp 35	PR110305m-	202	Dry	Ant exoeskeleton	EF371421.1	*Streptomyces vinaceus* strain 2255	2529	2529	97%
Streptomyces sp 35	PR110305m-	203	Dry	Ant exoeskeleton	EF371421.1	*Streptomyces vinaceus* strain 2255	2492	2492	97%
Streptomyces sp 36	PR110305m-	259	Dry	Ant exoeskeleton	NR_041116.1	*Streptomyces violarus* strain NBRC 13104	2490	2490	97%
Streptomyces sp 37	PR100815m-	42	Rainy	Ant exoeskeleton	FJ532421.1	*Streptomyces virginiae* strain HBUM175141	1279	1279	99%
Streptomyces sp 37	PR110305m-	287	Dry	Ant exoeskeleton	FJ481057.1	*Streptomyces virginiae* strain xsd08100	1471	1471	100%
Streptomyces sp 37	PR110305m-	293	Dry	Ant exoeskeleton	FJ481057.1	*Streptomyces virginiae* strain xsd08100	2473	2473	97%
Streptomyces sp 37	PR110305m-	295	Dry	Ant exoeskeleton	FJ481057.1	*Streptomyces virginiae* strain xsd08100	2464	2464	95%
Streptomyces sp 37	PR110305m-	299	Dry	Ant exoeskeleton	FJ481057.1	*Streptomyces virginiae* strain xsd08100	2473	2473	95%
Streptomyces sp 4	PR110305m-	201	Dry	Ant exoeskeleton	FJ532443.1	*Streptomyces aurantiacus* strain HBUM173139	1454	1454	97%
Streptomyces sp 41	PR110305m-	264	Dry	Ant exoeskeleton	FJ486295.1	*Streptomyces zaomyceticus* strain HBUM83735	1760	1760	96%
Streptomyces sp 5	PR110305m-	238	Dry	Ant exoeskeleton	EU593744.1	*Streptomyces aureus* strain 173978	1471	1471	100%
Streptomyces sp 8	PR110305m-	638	Dry	Ant exoeskeleton	FJ481059.1	*Streptomyces chartreusis*	2435	2435	95%
Streptomyces sp 9	PR110305m-	275	Dry	Ant exoeskeleton	NR_043344.1	*Streptomyces cinereoruber*	2440	2440	96%
Streptomyces sp 9	PR110305m-	282	Dry	Ant exoeskeleton	NR_043344.1	*Streptomyces cinereoruber*	2423	2423	94%
Streptomyces sp 9	PR110305m-	285	Dry	Ant exoeskeleton	NR_043344.1	*Streptomyces cinereoruber*	2473	2473	97%
Streptomyces sp 9	PR110305m-	634	Dry	Ant exoeskeleton	NR_043344.1	*Streptomyces cinereoruber*	2433	2433	95%
Tsukamurella sp1	PR100826m-	51	Rainy	Ant exoeskeleton	GU318217.1	*Tsukamurella tyrosinosolvens* strain E105	1260	1260	100%
Tsukamurella sp1	PR100826m-	54	Rainy	Ant exoeskeleton	AB480761.1	*Tsukamurella tyrosinosolvens* Acj 117	1434	1434	100%
Tsukamurella sp1	PR100826m-	65	Rainy	Ant exoeskeleton	AB478957.1	*Tsukamurella tyrosinosolvens* Acj 117	1249	1249	100%
Tsukamurella sp1	PR110305m-	213	Dry	Ant exoeskeleton	AB478956.1	*Tsukamurella tyrosinosolvens* Acj 117	2577	2577	98%
Kitasatospora sp 1	PR100815m-	6	Rainy	Yeast cultivar	NR_044810.1	*Kitasatospora kifunensis* strain JCM 9081	1334	1625	97%
Kitasatospora sp 2	PR100815m-	9	Rainy	Yeast cultivar	AF409019.1	*Kitasatospora* sp. Ellin177	1177	1177	100%
Kitasatospora sp 2	PR100815m-	104	Rainy	Yeast cultivar	AF409019.1	*Kitasatospora* sp. Ellin177	1441	1441	100%
Nocardia sp 1	PR110305m-	532	Dry	Yeast cultivar	EU741185.1	*Nocardia lijiangensis* strain 13658F	2213	2213	97%
Nocardia sp 2	PR110305m-	578	Dry	Yeast cultivar	GQ853079.1	*Nocardia niigatensis* strain W8186	2407	2407	97%
Rhodococcus sp2	PR100815m-	22	Rainy	Yeast cultivar	AB192962.1	*Rhodococcus opacus* strain:B-4	2536	2536	100%
Rhodococcus sp3	PR100815m-	16	Rainy	Yeast cultivar	FJ590420.1	*Rhodococcus wratislaviensis* strain IFP 2016	1149	1149	99%
Streptomyces sp 1	PR100815m-	96	Rainy	Yeast cultivar	AB184178.1	*Streptomyces aburaviensis* NBRC 12830	2490	2490	98%
Streptomyces sp 10	PR100826m-	76	Rainy	Yeast cultivar	EF654097.1	*Streptomyces drozdowiczii* strain NRRL B-24297	2580	2580	97%
Streptomyces sp 10	PR100826m-	84	Rainy	Yeast cultivar	EF654097.1	*Streptomyces drozdowiczii* strain NRRL B-24297	2573	2573	98%
Streptomyces sp 11	PR110305m-	562	Dry	Yeast cultivar	FJ532461.1	*Streptomyces exfoliatus* strain HBUM173195	2455	2455	95%
Streptomyces sp 14	PR110305m-	477	Dry	Yeast cultivar	GQ985452.1	*Streptomyces flavoviridis* strain ZG084	1818	1818	95%
Streptomyces sp 16	PR110305m-	529	Dry	Yeast cultivar	FJ532463.1	*Streptomyces gardneri* strain HBUM175034	2523	2523	97%
Streptomyces sp 17	PR110305m-	472	Dry	Yeast cultivar	AY314782.1	*Streptomyces glauciniger* strain FXJ14	2444	2444	95%
Streptomyces sp 18	PR110305m-	528	Dry	Yeast cultivar	AB184821.1	*Streptomyces griseus subsp.* rhodochrous	2527	2527	98%
Streptomyces sp 2	PR100815m-	5	Rainy	Yeast cultivar	AY999756.1	*Streptomyces albolongus* strain JCM 4716	2497	2497	97%
Streptomyces sp 22	PR100815m-	1	Rainy	Yeast cultivar	NR_043823.1	*Streptomyces kunmingensis* strain NRRL B-16240	2558	2558	99%
Streptomyces sp 22	PR100815m-	11	Rainy	Yeast cultivar	NR_043823.1	*Streptomyces kunmingensis* strain NRRL B-16240	2558	2558	99%
Streptomyces sp 22	PR100815m-	19	Rainy	Yeast cultivar	NR_043823.1	*Streptomyces kunmingensis* strain NRRL B-16240	2560	2560	99%
Streptomyces sp 23	PR110305m-	503	Dry	Yeast cultivar	GU479442.1	*Streptomyces lateritius* strain A25	2505	2505	97%
Streptomyces sp 24	PR110305m-	504	Dry	Yeast cultivar	JN585735.1	*Streptomyces omiyaensis* strain AC6	2388	2388	95%

Appendix A: Continuation

Streptomyces sp 24	PR110305m-	551	Dry	Yeast cultivar	AB184411.1	*Streptomyces omiyaensis* strain AC6	2479	2479	97%
Streptomyces sp 24	PR110305m-	553	Dry	Yeast cultivar	AB184411.1	*Streptomyces omiyaensis* strain AC6	2481	2481	97%
Streptomyces sp 25	PR100815m-	94	Rainy	Yeast cultivar	AB184515.1	*Streptomyces owasiensis* NBRC 13832	2577	2577	98%
Streptomyces sp 26	PR110305m-	502	Dry	Yeast cultivar	HQ607439.1	*Streptomyces phaeofaciens* strain 1187	2468	2468	97%
Streptomyces sp 26	PR110305m-	514	Dry	Yeast cultivar	HQ607439.1	*Streptomyces phaeofaciens* strain 1187	2431	2431	95%
Streptomyces sp 26	PR110305m-	599	Dry	Yeast cultivar	HQ607439.1	*Streptomyces phaeofaciens* strain 1187	2462	2462	97%
Streptomyces sp 27	PR100815m-	15	Rainy	Yeast cultivar	EU502867.1	*Streptomyces platensis* strain F160280	2425	2425	98%
Streptomyces sp 28	PR110305m-	530	Dry	Yeast cultivar	EU841669.1	*Streptomyces polychromogenes* strain HBUM174749	1498	1498	100%
Streptomyces sp 29	PR110305m-	495	Dry	Yeast cultivar	DQ462662.1	*Streptomyces pseudovenezuelae* strain B201	2438	2438	97%
Streptomyces sp 3	PR110305m-	498	Dry	Yeast cultivar	JN129837.1	*Streptomyces albus* strain LYT 1411	2409	2409	94%
Streptomyces sp 31	PR100815m-	14	Rainy	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2621	2621	100%
Streptomyces sp 31	PR110305m-	570	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	1509	1509	100%
Streptomyces sp 31	PR110305m-	571	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	1498	1498	100%
Streptomyces sp 31	PR110305m-	584	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	1458	1458	100%
Streptomyces sp 31	PR100815m-	92	Rainy	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2555	2555	97%
Streptomyces sp 31	PR110305m-	465	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2536	2536	97%
Streptomyces sp 31	PR110305m-	466	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2527	2527	97%
Streptomyces sp 31	PR110305m-	470	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2525	2525	97%
Streptomyces sp 31	PR110305m-	475	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2536	2536	97%
Streptomyces sp 31	PR110305m-	481	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2494	2494	97%
Streptomyces sp 31	PR110305m-	558	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	1467	1467	100%
Streptomyces sp 31	PR110305m-	567	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	1480	1480	100%
Streptomyces sp 31	PR110305m-	572	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2477	2477	95%
Streptomyces sp 31	PR110305m-	574	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2455	2455	95%
Streptomyces sp 31	PR110305m-	575	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2455	2455	95%
Streptomyces sp 31	PR110305m-	579	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2470	2470	95%
Streptomyces sp 31	PR110305m-	587	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2519	2519	97%
Streptomyces sp 31	PR110305m-	596	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2525	2525	97%
Streptomyces sp 31	PR110305m-	598	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2481	2481	95%
Streptomyces sp 31	PR110305m-	601	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2459	2459	95%
Streptomyces sp 31	PR110305m-	610	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2525	2525	98%
Streptomyces sp 31	PR110305m-	611	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2475	2475	95%
Streptomyces sp 31	PR110305m-	616	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2453	2453	95%
Streptomyces sp 31	PR110305m-	661	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2464	2464	95%
Streptomyces sp 31	PR110305m-	608	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2357	2357	96%
Streptomyces sp 31	PR110305m-	589	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	2298	2298	95%
Streptomyces sp 31	PR110305m-	583	Dry	Yeast cultivar	EU054375.1	*Streptomyces* sp. 8-1	1788	1788	94%
Streptomyces sp 33	PR100815m-	79	Rainy	Yeast cultivar	FJ481625.1	*Streptomyces tanashiensis* strain HBUM173179	2584	2584	98%
Streptomyces sp 33	PR110305m-	552	Dry	Yeast cultivar	EU841673.1	*Streptomyces tanashiensis* strain HBUM174095	1465	1465	100%
Streptomyces sp 38	PR100815m-	20	Rainy	Yeast cultivar	AY882020.1	*Streptomyces yanglinensis* strain 913	2431	2431	98%
Streptomyces sp 38	PR100815m-	26	Rainy	Yeast cultivar	AY882019.1	*Streptomyces yanglinensis* strain 317	2488	2488	98%
Streptomyces sp 38	PR100815m-	29	Rainy	Yeast cultivar	AY882019.1	*Streptomyces yanglinensis* strain 317	2449	2449	98%
Streptomyces sp 38	PR100815m-	32	Rainy	Yeast cultivar	NR_043244.1	*Streptomyces yanglinensis* strain 1307	2440	2440	100%
Streptomyces sp 38	PR100815m-	102	Rainy	Yeast cultivar	NR_043244.1	*Streptomyces yanglinensis* strain 1307	1122	1122	95%
Streptomyces sp 39	PR100826m-	105	Rainy	Yeast cultivar	NR_041427.1	*Streptomyces yatensis* strain NBRC 101000	2499	2499	99%
Streptomyces sp 40	PR100815m-	28	Rainy	Yeast cultivar	AB249943.1	*Streptomyces yeochonensis* NBRC 100782	2505	2505	100%
Streptomyces sp 40	PR100815m-	30	Rainy	Yeast cultivar	AB249943.1	*Streptomyces yeochonensis* NBRC 100782	2410	2410	99%
Streptomyces sp 41	PR100826m-	88	Rainy	Yeast cultivar	JN561301.1	*Streptomyces zaomyceticus* strain DSH-9	1376	1376	100%
Streptomyces sp 6	PR110305m-	500	Dry	Yeast cultivar	JN180215.1	*Streptomyces bungoensis* strain 15721	2447	2447	95%
Streptomyces sp 7	PR110305m-	511	Dry	Yeast cultivar	AB184453.2	*Streptomyces castaneus* NBRC 13670	2449	2449	97%

Appendix A: Continuation

Streptomyces sp 7	PR110305m-	516	Dry	Yeast cultivar	AB184453.2	*Streptomyces castaneus* NBRC 13670	2407	2407	97%
Streptomyces sp 8	PR100815m-	13	Rainy	Yeast cultivar	FJ481059.1	*Streptomyces chartreusis*	2719	2719	100%
Streptomyces sp 8	PR110305m-	527	Dry	Yeast cultivar	HQ607428.1	*Streptomyces chartreusis*	2486	2486	98%
Streptomyces sp 9	PR110305m-	483	Dry	Yeast cultivar	NR_043344.1	*Streptomyces cinereoruber*	2442	2442	97%
Streptomyces sp 9	PR110305m-	507	Dry	Yeast cultivar	NR_043344.1	*Streptomyces cinereoruber*	2470	2470	97%
Streptomyces sp 9	PR110305m-	534	Dry	Yeast cultivar	NR_043344.1	*Streptomyces cinereoruber*	2475	2475	97%
Streptomyces sp 9	PR110305m-	588	Dry	Yeast cultivar	NR_043344.1	*Streptomyces cinereoruber*	1461	1461	100%
Streptomyces sp 9	PR110305m-	612	Dry	Yeast cultivar	NR_043344.1	*Streptomyces cinereoruber*	2473	2473	96%
Tsukamurella sp1	PR100815m-	81	Rainy	Yeast cultivar	AB478956.1	*Tsukamurella tyrosinosolvens* Acj 117	2567	2567	99%
Kitasatospora sp 1	PR100815m-	6	1 Rainy	Yeast cultivar	NR_044810.1	*Kitasatospora kifunensis* strain JCM 9081	1334	1625	97%
Kitasatospora sp 2	PR100815m-	9	1 Rainy	Yeast cultivar	AF409019.1	*Kitasatospora* sp. Ellin177	1177	1177	100%
Kitasatospora sp 2	PR100815m-	104	1 Rainy	Yeast cultivar	AF409019.1	*Kitasatospora* sp. Ellin177	1441	1441	100%
Nocardia sp 1	PR110305m-	532	2 Dry	Yeast cultivar	EU741185.1	*Nocardia lijiangensis* strain 13658F	2213	2213	97%
Nocardia sp 2	PR110305m-	578	2 Dry	Yeast cultivar	GQ853079.1	*Nocardia niigatensis* strain W8186	2407	2407	97%
Rhodococcus sp2	PR100815m-	22	1 Rainy	Yeast cultivar	AB192962.1	*Rhodococcus opacus* strain:B-4	2536	2536	100%
Rhodococcus sp3	PR100815m-	16	1 Rainy	Yeast cultivar	FJ590420.1	*Rhodococcus wratislaviensis* strain IFP 2016	1149	1149	99%
Streptomyces sp 1	PR100815m-	96	1 Rainy	Yeast cultivar	AB184178.1	Streptomyces aburaviensis NBRC 12830	2490	2490	98%
Streptomyces sp 10	PR100826m-	76	1 Rainy	Yeast cultivar	EF654097.1	Streptomyces drozdowiczii strain NRRL B-24297	2580	2580	97%
Streptomyces sp 10	PR100826m-	84	1 Rainy	Yeast cultivar	EF654097.1	Streptomyces drozdowiczii strain NRRL B-24297	2573	2573	98%
Streptomyces sp 11	PR110305m-	562	2 Dry	Yeast cultivar	FJ532461.1	Streptomyces exfoliatus strain HBUM173195	2455	2455	95%
Streptomyces sp 14	PR110305m-	477	2 Dry	Yeast cultivar	GQ985452.1	Streptomyces flavoviridis strain ZG084	1818	1818	95%
Streptomyces sp 16	PR110305m-	529	2 Dry	Yeast cultivar	FJ532463.1	Streptomyces gardneri strain HBUM175034	2523	2523	97%
Streptomyces sp 17	PR110305m-	472	2 Dry	Yeast cultivar	AY314782.1	Streptomyces glauciniger strain FXJ14	2444	2444	95%
Streptomyces sp 18	PR110305m-	528	2 Dry	Yeast cultivar	AB184821.1	Streptomyces griseus subsp. rhodochrous	2527	2527	98%
Streptomyces sp 2	PR100815m-	5	1 Rainy	Yeast cultivar	AY999756.1	Streptomyces albolongus strain JCM 4716	2497	2497	97%
Streptomyces sp 22	PR100815m-	1	1 Rainy	Yeast cultivar	NR_043823.1	Streptomyces kunmingensis strain NRRL B-16240	2558	2558	99%
Streptomyces sp 22	PR100815m-	11	1 Rainy	Yeast cultivar	NR_043823.1	Streptomyces kunmingensis strain NRRL B-16240	2558	2558	99%
Streptomyces sp 22	PR100815m-	19	1 Rainy	Yeast cultivar	NR_043823.1	Streptomyces kunmingensis strain NRRL B-16240	2560	2560	99%
Streptomyces sp 23	PR110305m-	503	2 Dry	Yeast cultivar	GU479442.1	Streptomyces lateritius strain A25	2505	2505	97%
Streptomyces sp 24	PR110305m-	504	2 Dry	Yeast cultivar	JN585735.1	Streptomyces omiyaensis strain AC6	2388	2388	95%
Streptomyces sp 24	PR110305m-	551	2 Dry	Yeast cultivar	AB184411.1	Streptomyces omiyaensis strain AC6	2479	2479	97%
Streptomyces sp 24	PR110305m-	553	2 Dry	Yeast cultivar	AB184411.1	Streptomyces omiyaensis strain AC6	2481	2481	97%
Streptomyces sp 25	PR100815m-	94	1 Rainy	Yeast cultivar	AB184515.1	Streptomyces owasiensis NBRC 13832	2577	2577	98%
Streptomyces sp 26	PR110305m-	502	2 Dry	Yeast cultivar	HQ607439.1	Streptomyces phaeofaciens strain 1187	2468	2468	97%
Streptomyces sp 26	PR110305m-	514	2 Dry	Yeast cultivar	HQ607439.1	Streptomyces phaeofaciens strain 1187	2431	2431	95%
Streptomyces sp 26	PR110305m-	599	2 Dry	Yeast cultivar	HQ607439.1	Streptomyces phaeofaciens strain 1187	2462	2462	97%
Streptomyces sp 27	PR100815m-	15	1 Rainy	Yeast cultivar	EU502867.1	Streptomyces platensis strain F160280	2425	2425	98%
Streptomyces sp 28	PR110305m-	530	2 Dry	Yeast cultivar	EU841669.1	Streptomyces polychromogenes strain HBUM174749	1498	1498	100%
Streptomyces sp 29	PR110305m-	495	2 Dry	Yeast cultivar	DQ462662.1	Streptomyces pseudovenezuelae strain B201	2438	2438	97%
Streptomyces sp 3	PR110305m-	498	2 Dry	Yeast cultivar	JN129837.1	Streptomyces albus strain LYT 1411	2409	2409	94%
Streptomyces sp 31	PR100815m-	14	1 Rainy	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2621	2621	100%
Streptomyces sp 31	PR110305m-	**470**	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1443	1443	100%
Streptomyces sp 31	PR110305m-	570	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1509	1509	100%
Streptomyces sp 31	PR110305m-	571	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1498	1498	100%
Streptomyces sp 31	PR110305m-	584	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1458	1458	100%
Streptomyces sp 31	PR100815m-	92	1 Rainy	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2555	2555	97%
Streptomyces sp 31	PR110305m-	465	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2536	2536	97%
Streptomyces sp 31	PR110305m-	466	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2527	2527	97%
Streptomyces sp 31	PR110305m-	**470**	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2525	2525	97%
Streptomyces sp 31	PR110305m-	**475**	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2536	2536	97%

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Streptomyces sp 31	PR110305m-	**481**	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2494	2494	97%
Streptomyces sp 31	PR110305m-	558	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1467	1467	100%
Streptomyces sp 31	PR110305m-	567	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1480	1480	100%
Streptomyces sp 31	PR110305m-	572	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2477	2477	95%
Streptomyces sp 31	PR110305m-	574	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2455	2455	95%
Streptomyces sp 31	PR110305m-	575	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2455	2455	95%
Streptomyces sp 31	PR110305m-	579	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2470	2470	95%
Streptomyces sp 31	PR110305m-	587	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2519	2519	97%
Streptomyces sp 31	PR110305m-	596	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2525	2525	97%
Streptomyces sp 31	PR110305m-	598	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2481	2481	95%
Streptomyces sp 31	PR110305m-	601	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2459	2459	95%
Streptomyces sp 31	PR110305m-	610	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2525	2525	98%
Streptomyces sp 31	PR110305m-	611	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2475	2475	95%
Streptomyces sp 31	PR110305m-	616	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2453	2453	95%
Streptomyces sp 31	PR110305m-	661	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2464	2464	95%
Streptomyces sp 31	PR110305m-	608	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2357	2357	96%
Streptomyces sp 31	PR110305m-	589	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	2298	2298	95%
Streptomyces sp 31	PR110305m-	583	2 Dry	Yeast cultivar	EU054375.1	Streptomyces sp. 8-1	1788	1788	94%
Streptomyces sp 33	PR100815m-	79	1 Rainy	Yeast cultivar	FJ481625.1	Streptomyces tanashiensis strain HBUM173179	2584	2584	98%
Streptomyces sp 33	PR110305m-	552	2 Dry	Yeast cultivar	EU841673.1	Streptomyces tanashiensis strain HBUM174095	1465	1465	100%
Streptomyces sp 38	PR100815m-	20	1 Rainy	Yeast cultivar	AY882020.1	Streptomyces yanglinensis strain 913	2431	2431	98%
Streptomyces sp 38	PR100815m-	26	1 Rainy	Yeast cultivar	AY882019.1	Streptomyces yanglinensis strain 317	2488	2488	98%
Streptomyces sp 38	PR100815m-	29	1 Rainy	Yeast cultivar	AY882019.1	Streptomyces yanglinensis strain 317	2449	2449	98%
Streptomyces sp 38	PR100815m-	32	1 Rainy	Yeast cultivar	NR_043244.1	Streptomyces yanglinensis strain 1307	2440	2440	100%
Streptomyces sp 38	PR100815m-	102	1 Rainy	Yeast cultivar	NR_043244.1	Streptomyces yanglinensis strain 1307	1122	1122	95%
Streptomyces sp 39	PR100826m-	105	1 Rainy	Yeast cultivar	NR_041427.1	Streptomyces yatensis strain NBRC 101000	2499	2499	99%
Streptomyces sp 40	PR100815m-	28	1 Rainy	Yeast cultivar	AB249943.1	Streptomyces yeochonensis NBRC 100782	2505	2505	100%
Streptomyces sp 40	PR100815m-	30	1 Rainy	Yeast cultivar	AB249943.1	Streptomyces yeochonensis NBRC 100782	2410	2410	99%
Streptomyces sp 41	PR100826m-	88	1 Rainy	Yeast cultivar	JN561301.1	Streptomyces zaomyceticus strain DSH-9	1376	1376	100%
Streptomyces sp 6	PR110305m-	500	2 Dry	Yeast cultivar	JN180215.1	Streptomyces bungoensis strain 15721	2447	2447	95%
Streptomyces sp 7	PR110305m-	511	2 Dry	Yeast cultivar	AB184453.2	Streptomyces castaneus NBRC 13670	2449	2449	97%
Streptomyces sp 7	PR110305m-	516	2 Dry	Yeast cultivar	AB184453.2	Streptomyces castaneus NBRC 13670	2407	2407	97%
Streptomyces sp 8	PR100815m-	13	1 Rainy	Yeast cultivar	FJ481059.1	Streptomyces chartreusis	2719	2719	100%
Streptomyces sp 8	PR110305m-	527	2 Dry	Yeast cultivar	HQ607428.1	Streptomyces chartreusis	2486	2486	98%
Streptomyces sp 9	PR110305m-	483	2 Dry	Yeast cultivar	NR_043344.1	Streptomyces cinereoruber subsp. fructofermentans strain JC	2442	2442	97%
Streptomyces sp 9	PR110305m-	507	2 Dry	Yeast cultivar	NR_043344.1	Streptomyces cinereoruber subsp. fructofermentans strain JC	2470	2470	97%
Streptomyces sp 9	PR110305m-	534	2 Dry	Yeast cultivar	NR_043344.1	Streptomyces cinereoruber subsp. fructofermentans strain JC	2475	2475	97%
Streptomyces sp 9	PR110305m-	588	2 Dry	Yeast cultivar	NR_043344.1	Streptomyces cinereoruber subsp. fructofermentans strain JC	1461	1461	100%
Streptomyces sp 9	PR110305m-	612	2 Dry	Yeast cultivar	NR_043344.1	Streptomyces cinereoruber subsp. fructofermentans strain JC	2473	2473	96%
Tsukamurella sp1	PR100815m-	81	1 Rainy	Yeast cultivar	AB478956.1	Tsukamurella tyrosinosolvens Acj 117	2567	2567	99%