UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS - RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(ÁREA: MICROBIOLOGIA APLICADA)

FUNGI ASSOCIATED WITH *ACROMYRMEX* AND BASAL ATTINI ANTS FROM ARGENTINA AND BRASIL

VIRGINIA ELENA MASIULIONIS

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2013

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Tese apresentada ao Instituto de Biociências do campus de Rio Claro, Universidade Estadual Paulista "Julio de Mesquita Filho", como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas (Área: Microbiologia Aplicada).

Orientador: Prof. Dr. Fernando Carlos Pagnocca

Rio Claro

2013

«Wie alles sich zum Ganzen webt,

Eins in dem andern wirkt und lebt! »

(Goethe, 1749-1832, p.66)

« Através do telescópio Galileu confirmou a hipótese copernicana. O que ele perdeu foi o campo de movimento da astronomia vista ao olho nu, a relação da Via Láctea com o céu estrelado, e o movimento das jornadas de estrelas através do plano elíptico. E talvez em sua intensa concentração, ele tenha perdido também os sons, perfumes e cheiros da noite e a consciência de si mesmo como um homem que observa um esplêndido e misterioso espetáculo estelar. Galileu já não estava dentro da natureza, mas do lado de fora dela. Ele havia se tornado "observador científico". A natureza era agora um simples objeto de indagação científica »

(Oelschlaeger apub Grün, 2007, p.30)

Dedico este trabalho a Kostantinas, Germán, José, Elena, Agustín, Zinaida, Francisca e Antonio... Todos Eles minha Inspiração, Exemplo de Vida e Orgulho eterno... assim também, especialmente, a meu Orientador e Mentor o Prof. Fernando C. Pagnocca.

AGRADECIMENTOS

Devo confessar que nunca imaginei que faria meu doutorado aqui, no Brasil. Sinto que comecei meus estudos sendo um tipo de pessoa e quem está terminando... é alguém totalmente diferente... ficar e estudar aqui, foi uma das melhores experiências, senão a principal, da minha vida... obrigada a todos e cada um de vocês...

Além de dedicar este trabalho, agradeço a minha Família, que sem os ensinamentos e experiências que cada um deles me transmitirem e me transmitem constantemente, jamais poderia ter feito esse trabalho... só de pensar neles me cheio de orgulho, admiração, respeito... por isso, muito obrigada Kostantinas, Germán, José, Elena, Agustín, Zinaida, Francisca e Antonio... los amo!!

Agradeço e dedico este trabalho ao meu Orientador, o Prof. Fernando C. Pagnocca quem me deu a oportunidade de trabalhar no seu laboratório. Agradeço enormemente da ajuda que ele me deu quando eu cheguei aqui, o grande apoio, os conselhos e a estimulação constante que sempre recebe dele... as discussões sobre trabalhos, sobre ciência, sobre pesquisa...

Principalmente, agradeço ao Programa de Estudantes-Convênio de Pós-Graduação PEC-PG, administrado conjuntamente pelo Departamento Cultural (DP) do Ministério de Relações Exteriores - MRE, pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, pelo financiamento de meus estudos. Agradeço, também, à Universidade Estadual Paulista “Julio de Mesquita Filho” pela qualidade dos professores e do ensino.

Agradeço à Profa. Derlene Attili de Angelis por todo o apoio e ajuda que recebe dela quando cheguei a Rio Claro, assim como sua amizade.

Da seção de Pós-Graduação, agradeço pela enorme gentileza e paciência às adoráveis Rosemary Cardoso e Josiele Magri.

Agradeço enormemente ter as maravilhosas amizades (de valor incalculável para mim) de Lu (Luciana Fontes Coelho), Necis (mãe do bonitão Theo), “Ita” Reiss, Lu (Luciliene), Paula (Paulis) Sanchez e Ana Paula (Anis Juanis) Duarte.

Agradeço pela amizade, a meus colegas e amigos de laboratório!! Ife (uma pessoa que me insinuou muito), Silvio, Noemi, Weilan, Samuel (Samuelito), Francisco, Lídia,

Priscila, Juliana dos Santos, e aqueles que já não estão presentes mais estarão sempre comigo Thaís Mendes, Tati Carvalho, Aline Cruz, Aline Castanha, Fábio Guedes, Liu, Mara, Diogo, Marcela e Luiza.

Agradeço o bom e agradável relacionamento com o Prof. Odair Bueno, pelas boas conversações e conselhos ao Prof. Vanderlei Martins, pelos conselhos e o compartilhar ideias ao Prof. Hércules Menezes. Agradeço a gentileza e bom trato do Prof. André Rodrigues.

Não posso me esquecer de agradecer a excelente disposição para me ajudar e dar sugestões ao Prof. Silvio Govone, assim como as agradáveis conversações.

Agradeço pela gentileza e os bons conselhos que sempre me deu Sebastião Zanão. Também quero agradecer pela paciência e gostosas conversações a Antonio Teruyoshi Yabuki.

Do laboratório do Prof. Odair, nunca vou esquecer as amizades de Marcela (Marce), Vivi, Amandinha, Sandra (Sandrinha) e Eduardo (Du-du). Ao Prof. Osmar Malaspina (pelas pinhas!) a Andrigo e João. Do laboratório do Prof. Maurício Bacci Jr agradeço a gentileza de Milene, Joana (Jô!), Alexandre, Cynara, Carol, Sergio, Joaquim e Cíntia.

Agradeço a duas adoráveis e maravilhosas pessoas que considero minhas amigas de coração, Maria e Lídia pela amizade e as brincadeirinhas diárias! Elas sempre poderão contar comigo!

Do Departamento de Bioquímica agradeço a amizade de: Kate, Roberta, César, Alex, Fabrício, Túlio (Batata), Grazy, Paulo, Adriano; também a Rafinha (adorável!), Danilo, Sadala, Juliana, Mariana, Jaqueline e Lucas.

Ao pessoal da biblioteca!! Os quais são todos mega-legais, agradeço pela paciência e a excelente disposição para ajudar, adoro eles!

Agradeço, também, a Henrique e Laura, duas excelentes pessoas que me deram como presente a minha pequenininha amiga Mažutis.

Não quero me esquecer de agradecer a linda amizade que fiz com Ju Padilha com quem compartilhamos muitas e divertidas conversas, assim como também a minha outra amiga maravilhosa Cristina Costa Bombonatti com quem sorrimos o tempo tudo!

Agradeço muito especialmente a minha querida Professora de Português na Argentina, Lourdes Narvaez por ter uma excelente predisposição e paciência para me ensinar esta linda língua e que, se não fora por ela nunca poderia ter obtido esta bolsa de estudos.

Também gostaria de agradecer à Dra. Marta Cabello do Instituto Spegazzini, La Plata, Buenos Aires quem me aconselhou e ficou ao meu lado sempre. Ao Dr. Roberto Lecuona pela oportunidade de trabalhar no seu laboratório no IMyZA, INTA- Castellar, Buenos Aires, Argentina. A meus amigos do laboratório do IMyZA, Ana Laura (Anis Lauris, agora mãe), Julieta (Ju) e Ignácio (Nacho) pela ajuda incondicional, os momentos divertidos, as conversações, fofocas e "los mates de las mañanas y las tardes".

Ao Prof. Antonio Iozia, proprietário do campo onde trabalhei em Santurce, Santa Fé, por dois anos, ele sempre foi extremamente gentil e adorável comigo. Outra vez a meu amado irmão Germán por sua valiosa e indispensável amizade, companhia e ajuda incondicional.

Agradeço enormemente a fantástica amizade com o Dr. Roland W. Weber quem foi e é uma das pessoas com a qual mais me identifiquei no jeito de pensar a ciência.

Agradeço ao Dr. Richard Patrock por sua amizade que me fez sempre muito bem, ao Dr. Cameron R. Currie por estar sempre presente e por me aconselhar e estimular sempre, ao Dr. Urlich G. Mueller por me escutar, compreender e estimular a desenvolver determinados trabalhos.

Agradeço pelos conselhos ao Dr. Christian Rabeling e ao Dr. Henrik De Fine Licht, assim como também, gostaria de agradecer ao Dr. Ted Schultz e ao Dr. Maurício Bacci Jr pela participação no trabalho.

RESUMO

As formigas cultivadoras de fungo (tribo Attini) são insetos eusociais, os quais formam colônias de centenas até milhões de indivíduos, sendo o fungo a principal fonte de alimentação das larvas e a rainha. Segundo sua fungicultura, elas são divididas em grupos, onde encontram-se as denominadas agricultura de "Lower-Attini" (agricultura das formigas basais) e aquelas com agricultura derivada ou "Higher-Attini". Dentro da agricultura derivada, estão as formigas conhecidas como "cortadeiras", que cortam material vegetal fresco para utilizar como substrato para o fungo mutualista que cultivam, enquanto que as formigas com agricultura basal, usam como substrato exoesqueleto e fezes secas de insetos, sementes, material de serrapilheira e folhas secas. Dentro dos ninhos, constroem câmeras onde mantém o fungo formando uma estrutura em forma de esponja chamada de "jardim de fungo". O substrato é constantemente renovado e o material vegetal, uma vez tendo propiciado o crescimento do fungo é descartado, segundo a espécie de formiga, em câmaras especiais dentro do ninho ou externamente ao ar livre. Ainda, não se sabe muito sobre a estrutura e diversidade das comunidades de micro-organismos associados aos ninhos, apesar do crescente número de publicações a respeito. A biologia das formigas cortadeiras da Argentina é pouco estudada e praticamente não se sabe nada sobre os micro-organismos que encontram-se associados aos jardins de fungos e depósitos de descarte. Neste trabalho, apresentão-se dados sobre as leveduras que habitam e estão relacionadas com os ninhos de três espécies de formigas cortadeiras: *Acromyrmex heyeri*, *Acromyrmex lobicornis* e *Acromyrmex lundii*, assim como a descrição de duas espécies novas de leveduras. Mostram-se também um hábito de forrageamento incomum na espécie *A. lobicornis*. Ainda, estudando os jardins de fungo de duas formigas basais *Mycocepurus smithii* e *Mycocepurus goeldii* do Campus da UNESP, Rio Claro, Brasil, encontraram-se diferenças marcantes na morfologia do fungo cultivado por *M. smithii* e, pela primeira vez, apresenta-se e descreve-se uma espécie nova de um micoparasita específico pertencente ao gênero *Escovopsis* isolado de um jardim de *M. goeldii*. Acredita-se que os resultados aportam mais informação sobre a microbiota associada aos ninhos destas formigas.

PALAVRAS CHAVE: leveduras; diversidade; Attini; fungos filamentosos

RESUMEN

Las hormigas cultivadoras de hongos (Tribu Attini) son insectos eusociales, los cuales forman colonias de cientos hasta millones de individuos, siendo el hongo la principal fuente de alimentación de las larvas y la reina. Según, la fungicultura practicada, pueden ser divididas en grupos, donde se encuentran aquellas con agricultura conocida como "Lower-Attini" (agricultura de las hormigas basales) y aquellas con agricultura derivada o avanzada, "Higher-Attini". Dentro de la agricultura derivada, se encuentran las hormigas conocidas como "cortadoras o podadoras", las cuales cortan material vegetal fresco para utilizar como sustrato para el hongo mutualista que cultivan, mientras que las hormigas con agricultura de tipo basal, utilizan como sustrato exoesqueleto y excrementos secos de insectos, semillas, material de hojarasca y hojas secas. Dentro de los nidos, construyen cámaras donde mantienen el hongo formando una estructura semejante a una esponja denominada, comúnmente, como "jardín de hongo". El sustrato es constantemente renovado y el material vegetal, una vez consumido los nutrientes del mismo, es descartado en cámaras especiales dentro del nido, o bien, depositado exteriormente al aire libre, lo que depende de la especie de hormiga. Hasta ahora, no se sabe mucho sobre la estructura y diversidad de las comunidades de microorganismos que se encuentran asociados a los nidos, a pesar del creciente número de publicaciones hechas al respecto. La biología de las hormigas cortadoras de la Argentina ha sido poco estudiada y prácticamente no se sabe nada sobre los microorganismos que se encuentran asociados a los jardines de hongos y depósito de descarte. Con todo, este trabajo presenta datos sobre las levaduras que habitan y están relacionadas con los nidos de tres especies de hormigas podadoras: *Acromyrmex heyeri*, *Acromyrmex lobicornis* y *Acromyrmex lundii*, así como la descripción de dos especies nuevas de levaduras. También es mostrado, un habito de forrajeo poco común en la especie *A. lobicornis*. Además, estudiando los jardines de hongos de dos especies de hormigas basales *Mycocepurus smithii* y *Mycocepurus goeldii* del Campus de la UNESP, Rio Claro-Brasil, se han descubierto marcantes diferencias en la morfología del hongo cultivado por *M, smithii*. También, por primera vez, se presenta y describe una especie nueva de un conocido micoparásito específico perteneciente al género *Escovopsis* encontrada en el jardín de hongo de *M. goeldii*. Creemos que estos resultados pueden aportar un poco más de información sobre la micro-biota que se encuentra asociada a los nidos de estas hormigas.

PALABRAS LLAVES: levaduras; diversidad; Attini; hongos filamentosos

SUMÁRIO

1 INTRODUÇÃO

"Mais comment les mycelium de la moisissure se transforment-ils en «choux-raves» microscopiques? Quel est ce champignon mystérieux des *Attini*?"

(FOREL, 1923, p.66)

1.1 Primeiros registros

Dentre os insetos eusociais encontram-se as formigas da tribo Attini, as quais são cultivadoras de fungos. Dentre elas há um grupo conhecido como "formigas cortadeiras", o qual tem chamado a atenção através do tempo por ser considerada praga de diversas plantações e cultivos em geral (FOWLER et al., 1990). Entre as primeiras observações registradas sobre a existência dessas formigas e o dano que causavam, contam-se os de Gonzalo Fernández de Oviedo y Valdés em 1535 em sua obra "Historia General y Natural de las Indias" (1851) onde menciona os grandes prejuízos em plantações e a abundância destas formigas na Ilha Española (Santo Domingo).

Na América do Norte, Buckley (1860) foi o primeiro naturalista a publicar estudos sobre o habitat de *Atta texana* (*Œcodoma mexicana*, Sm), que pensava que essas formigas consumiam as folhas, as quais, segundo ele, eram trituradas formando uma esponja misturada com "secreções animais" (possivelmente fazia referência às hifas do fungo).

Em 1863, Bates, em seu clássico trabalho "The Naturalist on the River Amazon" descreve o trabalho na construção dos ninhos e forrageamento de folhas das "saúvas", *Atta cephalotes*, mas naquele momento desconhecia-se o motivo pelo qual essas formigas cortavam e carregavam folhas para o interior dos ninhos.

Anos mais tarde, em 1874, Thomas Belt, observando o comportamento de uma espécie de *Atta* escreveu sobre o uso das folhas: "Some naturalist have supposed that they use them directly as food; others, that they roof their underground nests with them. I believe the real use they make of them is as a manure, on which grows a minute species of fungus, on which they feed; that they are, in reality, mushroom growers and eaters". Belt também descreveu e denominou aquela esponja como "ant-food": "The chambers were always about

three parts filled with a speckled brown, flocculent, spongy-looking mass of a light and loosely connected substance. Throughout these masses were numerous ants belonging to the smallest division of the workers, and which do not engage in leaf-carrying. Along with them were pupae and larva, not gathered together, but dispersed, apparently irregularly, throughout the flocculent mass. This mass, which I have called the ant-food, proved, on examination, to be composed of minutely subdivided pieces of leaves, withered to a brown colour, and overgrown and lightly connected together by a minute white fungus that ramified in every direction throughout it" (BELT, 1874, p. 79).

Já em 1893, Möller em seu estudo com colônias de *Ac. disciger* descreveu detalhadamente a morfologia e crescimento do fungo cultivado, com o qual, somado a informação de Mc Cook (1879) denominaram "Pilzgärten" (em alemão significa "jardim de fungo") o que Belt havia chamado "ant-food".

Ao mesmo tempo, durante a detalhada descrição deste "jardim de fungo" de *Ac. disciger*, Möller (1893) observou e registrou a presença de grupos de hifas com os ápices entumescidos, aos quais ele deu o nome de "Kohlrabiknops" devido a sua similaridade com as cabeças de couve-rábano. Anos mais tarde, Wheeler (1907) escreveu: "As Mœller's terms for these structures are rather far-fetched, since to English-speaking peoples at least the kohlrabi is by no means a familiar vegetable, and as the structures really deserve somewhat more dignified or at any rate more technical appellations, I would suggest that the globular swellings of the hyphæ be called *gongylidia* and the grape-like clusters which they form, *bromatia*" (WHEELER, 1907, p. 739). *Gongilidium* (plural: *gongilidea*), derivado do grego *gongilis* que significa *nabo*. Weber (1957) renomeou ao cluster de *gongylidia* como *staphyla* (plural: *staphylae*) que em grego significa *conjunto de uvas*. Esta última característica do fingo cultivado, viria mais tarde a dividir o grupo das Attini em inferiores e superiores.

1.2 Formigas Attini — Fungos Basidiomicetos

1.2.1 Generalidades sobre as formigas cultivadoras de fungos

A localização sistemática deste grupo resulta em tribo Attini, subfamília Myrmicinae, família Formicidae, ordem Hymenoptera, classe Insecta (HÖLLDOBLER; WILSON, 1990). Atualmente, compreendem 15 gêneros com aproximadamente 296 espécies descritas (BRANDÃO et al., 2011). O número de espécies dentro de cada gênero é o seguinte:

Acromyrmex (62), *Apterostigma* (44), *Atta* (19), *Cyphomyrmex* (40), *Kalathomyrmex* (1), *Mycetagroicus* (4), *Mycetarotes* (4), *Mycetophylax* (3), *Mycetosoritis* (5), *Mycocepurus* (6), *Myrmicocrypta* (32), *Paramycetophylax* (1), *Pseudoatta* (1), *Serycomyrmex* (22) e *Trachymyrmex* (52) (BRANDÃO et al., 2011). A distribuição das Attini encontra-se restrita à região Neotropical, ocorrendo desde 40°N (New Jersey, Estados Unidos) até 44°S (Chubut, Argentina) (WHEELER, 1907; HÖLLDOBLER; WILSON, 2011).

Esta tribo é um grupo monofilético (grupos que incluem todos os taxa que descendem de um ancestral comum) de formigas conhecidas como "formigas cultivadoras de fungo" devido ao fato que elas cultivam fungos basidiomicetos com o qual tem uma relação simbiótica do tipo mutualista. Calcula-se que esta relação simbiótica se iniciou a 60-50 milhões de anos (CHAPELA et al., 1994; HINKLE et al., 1994; MUELLER et al., 1998; SCHULTZ; BRADY, 2008). O mutualismo é obrigatório porque ambos os parceiros são interdependentes, ou seja, um não ocorre na natureza sem a presença do outro (NORTH et al., 1997).

O fungo basidiomiceto (Lepiotaceae: Agaricales) da maioria das Attini derivadas apresenta uma característica particular a qual foi considerada como "único" marco evolutivo (SCHULTZ; BRADY, 2008), pois apresenta os ápices das hifas dilatados. Eles foram denominados "gongylidia" (gongilideos) e quando agrupados formam os denominados "staphylae", os quais são a fonte de alimento da rainha e das larvas, enquanto as operárias nutrem-se de modo misto, utilizando seiva das plantas, hifas e staphylae (MÖLLER, 1893; WEBER, 1957; LITTLEDYKE; CHERRETT, 1976; QUINLAN; CHERRETT, 1978, 1979; ANGELI-PAPA; EYMÉ, 1985; BASS; CHERRETT, 1995; MURAKAMI; HIGASHI, 1997). Estas estruturas contém no seu interior uma variedade de compostos e dentre eles os principais são açúcares tais como glucose, manitol, trealose, glicano, arabitol e glicogênio, além de lipídios e ergosterol (em menor medida) (MARTIN et al., 1969; QUINLAN; CHERRETT, 1978, 1979; MÔNACO FURLETTI; SERZEDELLO, 1983), e aminoácidos livres (MARTIN et al., 1969; HÖLLDOBLER; WILSON, 1990).

Dependendo da espécie de formiga, o fungo cresce sobre diversos tipos de substrato, como por exemplo, material vegetal fresco, exoesqueleto de insetos e sementes coletadas próximas aos ninhos (DE FINE LICHT; BOOMSMA, 2010), produzindo nutrientes e enzimas às formigas (MARTIN, 1970; RØNHEDE et al., 2004; SILVA et al., 2006; SCHIØTT et al., 2008; ERTHA Jr. et al., 2009; DE FINE LICHT et al., 2010). Por outro

lado, as formigas cuidam do jardim de fungos e evitam a proliferação de micro-organismos contaminantes através de diversas estratégias de higiene (LITTLE et al., 2003; LITTLE et al., 2006; MANGONE; CURRIE, 2007). Dentre elas destacam-se as descritas como "grooming" e "weeding" (CURRIE; STUART, 2001), além da adição de gotículas fecais, enzimas e secreções glandulares (MARTIN et al., 1973; FEVBAY; KERMARREC, 1981; FEVBAY et al., 1984; LIMA MENDOÇA et al., 2009).

Várias substancias com propriedades antibióticas são secretadas pelas glândulas metapleurais das formigas (ORTIUS-LECHNER et al., 2000; BOT et al., 2001; BOT et al., 2002; RODRIGUES et al., 2008) e por actinobactérias (CURRIE et al., 1999a; KOST et al., 2007; HAEDER et al., 2009; OH et al., 2009; BARKE et al., 2010), o que permite às formigas manter boas condições higiênicas, evitando a proliferação ou desenvolvimento de micro-organismos indesejáveis à simbiose (PAGNOCCA et al., 2012).

1.2.2 O fungo cultivado

O corpo de frutificação do fungo cultivado pelas Attini não é comumente encontrado na natureza. Em 1893, o botânico Möller foi o primeiro a descrever o basidiocarpo que cresceu sobre um ninho de *Ac. disciger* Mayr, no sul do Brasil. Este pesquisador, segundo a morfologia observada, o nomeou como *Rozites gongylophora*.

Em 1922, Spegazzini descreveu fungos que cresceram em ninhos velhos ou abandonados de *A. lundii*, acreditando que eles eram o fungo mutualista. Ele aplicou os nomes *Xylaria micrura* (ascocarpo maturo), *Bargellinia? belti* (hifas) e *Rhizomorpha formicarum* (estroma imaturo). Naquele momento ele narrou: "Entonces me convenci que la *Bargellinia*, la *Rhizomorpha* y la *Xylaria* no eran sino estados evolutivos de un mismo organismo y que las hifas micelianas de ápice hinchado, o góngilos, no eran exclusivas de los himenomicetas mirmecófilos, sino de todos los micélios cultivados por las hormigas de las diferentes familias; fácilmente, las hifas sometidas a constantes y sucesivas esquilas, toman todas, por la influencia de uma misma causa excitadora, caracteres morfológicos parecidos, y, por lo tanto, todos los hongos mirmecófilos a cualquier familia que pertenezcan resultan gongilóforos" (SPEGAZZINI, 1922, p.170-171). Spegazzini (1922) também descreveu os estromas de *Locellina mazzuchii* crescendo no ninho de *A. vollenweideri* no norte da Argentina e *Poroniopsis bruchi* isolado a partir de substrato velho de um ninho de *A. heyeri.* Wheeler (1907) aplicou um outro nome ao fungo que encontrou

em um ninho de *Cyphomyrmex comalensis* e escreveu: “In the meantime the singular fungus cultivated by *C. comalensis* and the other forms of *rimosus* over such an extensive area of the American tropics certainly deserves a name, and even at the risk of creating a synonym, I propose to call it *Tyridiomyces formicarum* gen. et sp. nov. and to assign it provisionally to the order Exoaceae, a group which also includes the well-known yeast fungi” (WHEELER, 1907, p. 772).

Em 1957, Weber observou pela primeira vez, o corpo de frutificação em um ninho de *Cyphomyrmex costatus* do Panamá, o qual foi identificado como pertencendo ao gênero *Leucocoprinus*. Heim (1957, p.289) escreveu: “On peut d’autant mieux l’affirmer que la comparaison des caractères et des protographies de l’espèce de Möller e de celle de Weber fait apparaître leur très vraisemblable identité” e com isso, ele denominou como *Leucocoprinus gongylophora*.

Kreisel (1972) examinando o jardim de fungo associado a *A. insularis* de Cuba escreveu “Aus der mikroskopischen Untersuchung ging hervor, daß die Pilzgärten ausschließlich von dem nachstehend als *Attamyces bromatificus* beschriebenen Pilz besiedelt waren. Bromatien waren stets reichlich vorhanden. Andere Pilze fehlten.”, e descreve assim como a espécie *Attamyces bromatificus*. É importante ressaltar que a esta altura havia várias sinonímias para um único organismo e até mesmo fungos não mutualistas foram considerados como tal, conforme descrito acima em relação a Spegazzini. Uma das razões que dificultaram uma melhor identificação do fungo mutualista foi a dificuldade natural de se encontrar a fase sexuada (basidioma), a qual é fundamental para a identificação taxonômica mediante os métodos tradicionais.

Muchovej et al. (1991) denominaram de *Leucoagaricus weberi* ao fungo encontrado em ninhos de *A. sexdens rubropilosa*. Pagnocca et al. (2001) coletaram basidiocarpos encontrados em um ninho de *A. hyspidus falax*, o qual também foi identificado como sendo *Leucoagaricus gongylophorus;* nesse momento, utilizando a emergente técnica molecular conhecida como RAPD (Random Amplification of Polymorphic DNA) esses autores puderam confirmar pela primeira vez que não se tratava de contaminação, pois tanto o tecido micelial como o basidiocarpo eram geneticamente iguais.

Atualmente, com o desenvolvimento das técnicas moleculares e das análises filogenéticas se pode constatar que os fungos pertencem à família Lepiotaceae (Basidiomycota: ordem Agaricales) localizado dentro da tribo Leucocoprinae (CHAPELA et

al., 1994; HINKLE et al., 1994; MUELLER et al., 1998) a qual contém os gêneros *Leucocoprinus* e *Leucoagaricus*. Apesar de que a maioria das Attini cultiva fungos da família Lepiotaceae, as do gênero *Apterostigma* cultivam fungos das famílias Lepiotaceae e Pterulaceae, próximamente relacionados à família Tricholomataceae dentro da mesma ordem Agaricales (CHAPELA et al., 1994).

1.3 Ciclo de vida das formigas Attini

> "Mais plus Huber a réussi à voir comment Madame *Atta* alimente *directement* sés larves avec lês œufs qu'elle pond"
>
> (FOREL, 1923, p.89)

O ciclo de vida de uma colônia, assim como o ciclo de vida de uma formiga, pode ser dividido em três estados: (i) fundação da colônia, (ii) estado ergonômico (estado de crescimento relativamente rápido da colônia no qual são produzidos só operárias) e (iii) estado reprodutivo (HÖLLDOBLER; WILSON, 1990).

Nas Attini derivadas o fungo é transmitido de geração em geração pela casta real dando começo assim a uma nova colônia. Von Ihering (1898) mostrou que as fêmeas virgens (futuras rainhas) da espécie *A. sexdens*, ao deixar o ninho para empreender o vôo nupcial, levam na cavidade infrabucal (cavidade na base da câmara bucal na qual o material não ingerido é acumulado e compactado para logo ser eliminado) um pequeno pedaço (pellet) de micélio retirado do jardim de fungo maternal. Depois do acasalamento que aparentemente acontece no vôo nupcial, a fêmea fecundada retorna ao solo e se desfaz das suas asas; rapidamente, começa a preparar uma cavidade no solo que continua por uma galeria e termina em uma câmara. Ela fecha a entrada do ninho e assim permanece enclausurada por algum tempo (HUBER, 1908; BRUCH, 1916; BRUCH, 1922; HÖLLDOBLER; WILSON, 1990).

Dentro da câmara, a rainha deposita o pequeno pellet de micélio e passa a cultivá-lo, nutrindo-o com pequenas gotas de líquido fecal; ao mesmo tempo, começa a pôr ovos e cuidar das larvas (HUBER, 1908). Nesta etapa a rainha produz ovos tróficos, os quais utiliza para sua própria alimentação e das primeiras larvas (HUBER, 1908).

Este comportamento, embora bastante generalizado entre as Attini, apresenta exceções, pois já foi observado que a rainha de *Acromyrmex lundii* pode atuar também como operária durante este período, saindo às vezes do ninho para coletar fragmentos de vegetais (BRUCH, 1919, 1922; BONETTO, 1959).

Todo o explicado acima, acontece nas formigas com reprodução sexual, já que naquelas que se reproduzem por partenogênese telítoca como é o caso de *Mycocepurus smithii* (RABELING et al., 2011), ainda não é conhecido o ciclo de vida da colônia.

1.4 Forrageamento, seleção e processamento do substrato

O forrageamento (WILSON, 1953, 1980) implica a busca, seleção, corte e transporte do material vegetal (folhas, flores, frutos, sementes) (WHEELER, 1907; WEBER, 1972) para dentro do ninho (HÖLLDOBLER; WILSON, 1990; DELLA LUCIA; OLIVEIRA, 1993; RAMOS RIBEIRO; SANTOS MARINHO, 2011). Este comportamento é amplamente estudado nas formigas dos gêneros *Atta* e *Acromyrmex* sendo menos estudado nas Attini inferiores.

O material vegetal é cuidadosamente selecionado pelas operárias e esta seleção depende de parâmetros físicos tais como: a dureza e conteúdo de água das folhas (BOWERS; PORTER, 1981; WALLER, 1982; NICHOLS-ORIANS; SCHULTZ, 1989) e a composição química das folhas para evitar toxinas, compostos secundários, terpenóides ou compostos antifúngicos (CHERRETT, 1972; ROCKWOOD, 1975, 1976; LITTLEDYKE; CHERRETT, 1978; HUBBELL et al., 1984; HOWARD, 1988). A preferência pelo forrageamento sobre plantas monocotiledôneas (formigas cortadeiras de grama) ou dicotiledôneas, depende da espécie de formiga, embora algumas espécies possam forragear sobre ambos os tipos de plantas (FOWLER et al., 1990; FRANZEL; FARJI-BRENER, 2000; LOPES, 2005). Dentro do ninho, o substrato é distribuído pelas diferentes câmaras onde é processado (MOREIRA et al., 2004a; MOREIRA et al., 2004b). O processamento implica um conjunto de comportamentos desenvolvidos pelas diferentes castas com a finalidade de eliminar contaminantes e promover a colonização inicial do substrato (WILSON, 1980; HÖLLDOBLER; WILSON, 1990). O substrato é lambido, triturado, fragmentado e ao mesmo tempo, tratado com enzimas digestivas para finalmente incorporá-

lo ao jardim de fungo pré-existente (HÖLLDOBLER; WILSON, 1990; ANDRADE et al., 2002; RØNHEDE et al., 2004; DINIZ; BUENO, 2009).

1.5 Sistemas de Fungicultura

Segundo Schultz e Brady (2008) e Mehdiabadi e Schultz (2009), a fungicultura das formigas Attini está dividida em 5 categorias: (I) agricultura basal, (II) agricultura "coral fungus", (III) agricultura de leveduras, (IV) agricultura derivada generalizada e (V) agricultura derivada das "formigas cortadeiras".

I. Agricultura basal. Dentro deste grupo se encontram os seguintes dez gêneros de formigas: *Myrmicocrypta*, *Mycocepurus*, *Kalathomyrmex*, *Paramycetophylax*, *Apterostigma* (*A. auriculatum*), *Mycetophylax*, *Mycetarotes*, algumas *Cyphomyrmex* (grupos *C. strigatus* e *C. wheeleri*), *Mycetosoritis* e *Mycetagroicus*. O fungo cultivado por este grupo pertence à tribo Leucocoprinae, estreitamente relacionada à família Lepiotaceae (Agaricales: Basidiomycota) embora tenham sido domesticados outros fungos localizados taxonomicamente dentro de outras famílias (CHAPELA et al. 1994; MUELLER, 2002). Este grupo de fungos é considerado dentro do grupo nomeado como G3 (CHAPELA et al. 1994; MUELLER, 2002). O principal substrato coletado são restos de artrópodes (WEBER, 1972; SCHULTZ; MEIER, 1995; MUELLER et al., 1998) mas foi observado que durante a estação úmida coletam flores e frutos, e, durante a estação seca, coletam fezes e cadáveres de insetos (LEAL; OLIVEIRA, 2000).

II. Agricultura "Coral fungi". Encontra-se representado pelo grupo *Apterostigma pilosum*. Este grupo cultiva fungos da família Pterulaceae, classificados como G2 ou G4 (MUELLER, 2002). O material forrageado por estas formigas para ser utilizado como substrato são fezes de insetos, sementes, partes de flores, pedaços de madeira e frutos (SCHULTZ; BRADY, 2008; MEHDIABADI; SCHULTZ, 2009). O gênero *Apterostigma* está dividido em dois clados, sendo que um deles está relacionado com *A. pilosum* (formiga cultivadora de "coral fungus") e o segundo clado é formado pelas formigas basais *A. auriculatum*, as quais cultivam fungos da tribo Leucocoprineae.

III. Agricultura de "yeast-balls": (bolotas de leveduras). Este grupo está representado pelo gênero *Cyphomyrmex* dividido em dois grupos ou "complexo de espécies": *C. strigatus* e *C. rimosus*. Segundo Schultz e Brady (2008) um terceiro grupo, *C. wheeleri*, deveria

também ser considerado em separado, mas encontra-se dentro do grupo *C. rimosus* (KEMPF, 1965). *C. rimosus* cultiva o fungo em forma de pequenos nódulos de leveduras, de cor amarelada que se confundem com grânulos de areia. Na verdade, essas bolotas encontra-se um fungo basidiomiceto leucocoprinaceo do grupo G3 (CHAPELA et al., 1994; SCHULTZ; BRADY, 2008) e Clado-1 (MUELLER et al., 1998) que ocorre sob duas morfologias (unicelular, como levedura e multicelular, como micélio). O material que é utilizado como substrato para este fungo são exosqueletos de insetos, seiva de plantas e néctar (MEHDIABADI; SCHULTZ, 2009). Por outro lado, os grupos de *C. strigatus* e *C. wheeleri* praticam uma agricultura basal.

IV. Agricultura derivada generalizada. Dentro da agricultura derivada encontram-se dois grupos bem diferenciados de formigas. O grupo composto pelos gêneros *Trachymyrmex* e *Sericomyrmex* que praticam a agricultura derivada generalizada e o segundo grupo conformado por *Atta* e *Acromyrmex* que são as conhecidas "formigas cortadeiras" (tratadas a seguir). Segundo Schultz e Brady (2008), este grupo está formado por 3 clados: (1) "Clado *Serycomyrmex*" consistindo do gênero *Serycomyrmex* (grupo monofilético) e espécies de *Trachymyrmex* (*T. opulentus*, *T. jamaicensis*, *T. urichii*, *T. iheringi*); (2) "Clado *T. intermedius*" formado por *T. intermedius*, *T. diversus*, *T. cortnezi*, *T. bugnioni* e (3) "Clado *T. septentrionalis*" que inclui *T. septentrionalis* e outras espécies relacionadas da América do Norte. Este grupo de formigas utiliza exoesqueleto de insetos, sementes, flores e frutos como substrato para o fungo mutualista (MUELLER, 2002). O fungo cultivado tanto por *Trachymyrmex* e *Sericomyrmex* assim como por *Atta* e *Acromyrmex*, pertence à tribo Leucocoprineae, família Lepiotaceae e é classificado como grupo G1 (CHAPELA et al., 1994; MUELLER, 2002; SCHULTZ; BRADY, 2008).

V. Agricultura derivada das "formigas cortadeiras". As "formigas cortadeiras" propriamente ditas estão representadas pelos gêneros *Atta* ("saúvas") e *Acromyrmex* ("quenquéns"). A característica principal que diferencia este grupo é a utilização preferencial de material vegetal fresco, principalmente folhas, flores, frutos como substrato para o fungo, embora sementes e partes vegetais secas também sejam forrageadas. Dependendo da espécie de formiga, a preferência pode recair tanto sobre plantas monocotiledôneas como dicotiledôneas. (WEBER, 1972; MUELLER et al., 1998; SCHULTZ; BRADY, 2008). Este grupo é relativamente jovem, tendo se originado entre 10 e 8 milhões de anos atrás (SCHULTZ; BRADY, 2008).

1.6 Modelos e hipóteses sobre a origem do mutualismo

Segundo, Leigh (2010), o mutualismo entre espécies envolve a troca de bens e serviços, os quais refletem as atitudes particulares das espécies que os proporcionam. Tendo em conta esta definição, são propostos dois modelos e sete hipóteses que tentam explicar a origem desta relação mutualista entre os fungos e as Attini.

1.6.1 Modelos

Existem dois modelos teóricos expostos claramente no trabalho de Mueller et al. (2001) que explicam a possível origem da domesticação do fungo mutualista. Um deles é o proposto por Weber (1972) conhecido como o "Traditional Consumption First" o qual propõe que "fungos não especializados" formaram parte da dieta das formigas, talvez porque cresceram acidentalmente nos ninhos (Consumption). Em seguida, as formigas desenvolveram estratégias para cultivá-los, selecionando substratos adequados (Cultivation) e, por último, encontraram o meio pelo qual o fungo podia ser transmitido de geração em geração (Transmission). O outro modelo é o "Alternative Transmission First" que assume que as formigas não consumiam diretamente o fungo; ao contrário, o fungo utilizaria as formigas como vetores para sua dispersão (Transmission); logo as formigas adotariam este fungo como parte de sua dieta (Consumption) e finalmente desenvolveram a capacidade de cultivá-lo.

1.6.2 Hipóteses

Conhecem-se sete hipóteses sobre a origem da fungicultura (MUELLER et al., 2001): (1) sementes armazenadas, (2) fungo nas paredes do ninho, (3) madeira em decomposição, (4) micorrizas, (5) cadáveres de artrópodes e pilhas de lixo, (6) fezes de formigas e (7) pellets infrabucais.

Sementes armazenadas. Von Ihering (ver WEBER, 1972) sugeriu que as Attini evoluíram de formigas coletoras de sementes e que a primeira formiga Attini encontrou o fungo mutualista como um contaminante.

Fungo nas paredes do ninho. Esta hipótese sugere que as Attini começaram cultivando um fungo que crescia sobre as paredes dos ninhos ancestrais.

Madeira decomposta. Forel (1891) postula que o grupo irmão das Attini é o Dacetini extrapolando para o ancestral das Attini, o comportamento de *Strumigenys* (pertencente a Dacetini) de construir ninhos na madeira decomposta, explicando que as Attini ancestrais puderam construir seus ninhos do mesmo jeito e se alimentar de fungos que cresceram na madeira decomposta.

Micorrizas. Garling (1979) sugeriu que as Attini adotaram um fungo tipo micorriza associado às raízes de plantas.

Cadáveres de artrópodes e pilhas de lixo. Propõe-se que o fungo mutualista pode ter sua origem de fungos que cresceram sobre operárias mortas ou larvas ou de presas descartadas nos depósitos de lixo.

Fezes de formigas. Forel (1902) propôs que as formigas evoluíram de ancestrais predadores que habitavam em madeira podre e começaram a se alimentar de fungos coprófilos de fezes de insetos que furavam a madeira. Anos mais tarde, Wheeler (1907) muda a hipóteses de fezes de insetos propondo fezes de formiga tendo em conta que as rainhas de *Atta* adubam o jardim de fungo com secreções fecais.

Pellets infrabucais. Em base ao estudo do conteúdo de conídios de diversos fungos encontrados nos pellets transportados pelas rainhas, Bailey (1920) sugeriu que a fungicultura teria ocorrido como um evento secundário onde as formigas seriam usadas pelo fungo como vetores para a própria dispersão.

1.7 “Microcosmos”: Os ninhos das formigas Attini

Nos estudos iniciais se pensou que as formigas Attini mantinham seus jardins de fungo em condições axênicas (cultivos puros). Porém, os jardins contêm variedades de outros micro-organismos como leveduras, fungos filamentosos e bactérias com diferentes tipos de associação onde poderiam ser mutualistas, parasitas ou oportunistas.

1.7.1 Leveduras

As leveduras podem ser encontradas tanto no ninho (jardim e deposito de descarte), assim como, sobre o exoesqueleto dos membros da colônia.

(a) *Leveduras isoladas do jardim e depósito de material descartado*

Pouco se sabe sobre a população de leveduras presentes e seu papel biológico na interação formiga-fungo mutualista. Craven et al. (1970) isolaram leveduras dos jardins de fungo de ninhos de laboratório de *Atta cephalotes* e de *Acromyrmex octospinosus,* mas não as identificaram. Carreiro et al. (1997) encontraram várias leveduras associadas a ninhos de laboratório de *Atta sexdens rubropilosa*, entre as quais as espécies dominantes foram fenotipicamente identificadas como *Candida homilentoma*, *Debaryomyces hansenii* e *Torulaspora delbrueckii*. Também, há registro de isolamento de leveduras do depósito de material descartado, que é o material descartado pelas operárias, tais como *Meyerozyma guilliermondii*, *Rhodotorula glutinis*, *Trichosporon beigelii* (PAGNOCCA et al., 1996; CARREIRO et al., 1997). Posteriormente, Middelhoven et al. (2003) e Carreiro et al. (2004) descreveram duas novas espécies encontradas nos jardins de fungo: *Cryptococcus haglerorum* e *Blastobotrys attinorum*, respectivamente.

Pagnocca et al. (2008) trabalhando com fêmeas aladas (içás virgens) das espécies *Atta laevigata* e *Atta capiguara* isolaram leveduras da cavidade infrabucal e do exoesqueleto, mostrando que vários micro-organismos, além do fungo simbionte, são dispersados durante a revoada.

Pagnocca et al. (2010) descreveram pela primeira vez a composição de espécies de leveduras encontradas no jardim de fungo e no depósito de lixo de uma espécie até então não descrita de *Myrmicocrypta*, *M. camargoii* (SOZA; CALVO, 2010) encontrada em Botucatu, SP, Brasil. Ao lado de espécies tais como *Hanseniaspora uvarum*, *Candida oleophila*, *Candida dubliniensis* e *Cryptococcus haglerorum*, foi descrita uma nova espécie encontrada maciçamente nessas amostras, *Trichosporon chiarellii* (PAGNOCCA et al., 2010). A razão desta espécie predominar neste ninho permanece desconhecida, pois apenas um ninho desta Attini foi encontrado até o momento.

O papel das leveduras na simbiose não é claro, mas foi sugerido que a habilidade de muitas leveduras em degradar alguns polissacarídeos de plantas pode contribuir com a disponibilidade de nutrientes para o fungo simbionte (CARREIRO, 2000). Mendes et al. (2012) mostraram que as leveduras, assim como observado por Ribeiro (2000) com as bactérias, poderiam ter uma participação importante no jardim de fungos pela atividade hidrolítica sobre os polissacarídeos vegetais. Além de gerar nutrientes de fácil assimilação para elas, para o fungo mutualista, para as formigas e demais micro-organismos presentes, essas leveduras também podem assimilar o ácido galacturônico. O fungo mutualista e as leveduras produzem pectinase e presume-se (SIQUEIRA et al., 1998) que isso resulte em grande quantidade de ácido galacturônico. Este composto afeta negativamente as formigas e não é utilizado pelo fungo mutualista como fonte de carbono (SILVA et al., 2003). O ácido galacturônico não se acumula nos ninhos pois muitas espécies de leveduras que ocorrem nos ninhos o utilizam como nutriente e com isso parece que contribuem para a sobrevivência dos simbiontes, eliminando um composto prejudicial a eles.

Carreiro et al. (2002) encontraram em ninhos de *A. sexdens*, leveduras que secretam micocinas ou toxinas “killer” as quais são proteínas de baixo peso molecular que inibiram o crescimento de outras leveduras isoladas do mesmo ambiente, bem como leveduras de outras origens. Os autores sugerem que algumas espécies de leveduras encontram no ambiente dos ninhos, especialmente na massa micelial e no depósito de descarte, locais propícios para sua sobrevivência e multiplicação e que as espécies dotadas de micocinas tipo “killer” podem exercer algum controle sobre as espécies não resistentes a ação dessas micocinas.

Rodrigues et al. (2009) mostraram mediante ensaios *in vitro* que, algumas leveduras encontradas nos jardins de quatro ninhos de *A. texana* inibiram o crescimento micelial de fungos, tais como *Syncephalastrum racemosum* (comumente isolado), assim como também, *Escovopsis* sp. e o fungo entomopatogênico *Beauveria bassiana*, e os autores propõem que as leveduras podem contribuir à proteção dos ninhos através desta atividade antagônica para com esses fungos.

Mendes et al. (2012), trabalhando com os jardins de fungos de oito espécies de *Acromyrmex* e *A. texana*, mostraram que as leveduras possuem atividade enzimática com capacidade de quebrar os polissacarídeos vegetais que são encontrados no substrato onde é inoculado o fungo.

(b) *Relacionadas com o corpo das formigas*

Com relação ao exosqueleto das formigas, Little e Currie (2007, 2008) isolaram e identificaram uma levedura negra ("black yeast") do corpo da formiga *Apterostigma* sp como pertencente ao gênero *Phialophora* sp. (Ascomycota). Este tipo de levedura cresce dentro de "fóveas" (pequenos poços na superfície do corpo da formiga) na cutícula das formigas, associada às actinobactérias, tais como, as pertencentes ao gênero *Pseudonocardia* sp. Os autores propõem que esta levedura atuaria como antagonista da actinobácteria, inibindo a capacidade das formigas de suprimir o crescimento do micoparasita *Escovopsis* (LITTLE; CURRIE 2008).

Não unicamente leveduras negras, como *Phyalophora*, se encontram no exoesqueleto das formigas senão também se encontram outros microfungos. Mais recentemente, Guedes et al. (2012) isolaram do exoesqueleto de operárias de *A. laevigata*, fungos tais como *Alternaria arborescens*, *Bipolaris sorokiniana*, *Bipolaris eleusines*, *Bipolaris zeae*, *Curvularia trifolii* e *Paraphaeosphaeria michotii***,** alguns deles conhecidos como fitopatógenos.

1.7.2 Fungos filamentosos

O trabalho de Möller (1893) com ninhos de *A. disciger* descreveu a presença de fungos filamentosos dos gêneros *Rhizopus*, *Aspergillus*, *Mucor* e *Penicillium* no jardim de fungo. Assim, os mesmos gêneros de fungos e alguns outros como *Cunninghamella*, *Fusarium*, *Trichoderma*, *Cladosporium* e *Nigrospora* foram observados indistintamente em jardins de fungos de *A. insularis*, *T. septentrionalis* e *A. heyeri* (GOETSCH; STOPPEL, 1940; WEBER, 1955; KREISEL, 1972; BASS; CHERRETT, 1994; LUCIANO, 1995; BARBOSA, 2004).

Em trabalhos realizados com ninhos de *Atta cephalotes*, os micro-organismos isolados foram aqueles comumente encontrados nas folhas carregadas pelas operárias, assim como também se observou que a estrutura da comunidade dos fungos mudava dependendo do tipo de dieta à qual eram submetidas às formigas (FISHER et al., 1996). Rodrigues et al. (2005a, 2008) trabalhando com os jardins das formigas *Atta* e *Acromyrmex* isolaram gêneros de microfungos encontrados habitualmente no solo, como por exemplo, *Syncephalastrum racemosum*, *Trichoderma harzianum* e *Cunninghamella* (RODRIGUES et al., 2005b).

Poulsen e Currie (2006) propõem que os fungos estão presentes nos jardins em forma de esporos.

1.7.3 Micoparasitismo

O parasitismo é uma relação interativa entre dois organismos ou populações na qual uma das partes prejudica a outra, enquanto a outra se beneficia (ATLAS; BARTHA, 2005). O micoparasitismo envolve a interação parasita entre dois fungos.

Möller (1893), estudando o jardim de fungos das formigas *A. disciger* e *Apterostigma* em Blumenau (Brasil), observou e descreveu em detalhe a presença de dois tipos diferentes de fungos que naquele momento ele pensou que eram os estados anamórficos (forma asexuada) dos fungos basidiomicetos cultivados pelas formigas. Kreisel, em 1972, redescobriu um dos fungos observado e registrado por Möller (1893) em um ninho de *Atta insularis* em Cuba. Ele o descreve formalmente e o denomina *Phialocladus zsoltii*. Entretanto, analisando a descrição feita por Kreisel (1972), Muchovej e Della Lucia (1990), baseando-se no artigo Nº 37 do Código Internacional de Nomenclatura Botânica sugeriram a mudança do nome do gênero para *Escovopsis,* propondo para a espécie a denominação *Escovopsis weberi* (em homenagem ao mirmecólogo Neil Weber).

Voltando ao princípio, o segundo tipo de fungo observado por Möller (1893) no jardim de fungo de espécies de formigas *Apterostigma* (*Ap. wasmannii*, *Ap. pilosa* e *Ap. moelleri*) foi por ele descrito como uma morfologia similar ao fungo *Aspergillus*, mas não o nomeou formalmente. Em 1995, Seifert et al. redescobriram este segundo fungo mas o encontra associado a jardins da espécie da formiga *Trachymyrmex ruthae* de Trinidad, descrevendo-o como *Escovopsis aspergilloides* devido a sua similaridade com as espécies de fungos do gênero *Aspergillus*.

Até aquele momento, não se sabia a possível função desse fungo no jardim, mas Currie et al. (1999b) isolaram *E. weberi* do jardim de *Aperostigma*, *Cyphomyrmex*, *Trachymyrmex*, *Acromyrmex* e *Atta*, os anteriores proposeram que este fungo era um micoparasita específico do fungo cultivado pelas formigas cortadeiras. Isso baseado no fato que, membros da mesma ordem Hypocreales são comumente micoparasitas ou micosapróbicos, os quais são extremadamente versáteis na sua habilidade em aproveitar o fungo como substrato (GAMS et al., 2004).

Segundo Currie et al (2001b), *Escovopsis* sp. é um micoparasita necrotrófico que, aparentemente, se nutre das hifas do fungo simbionte (REYNOLDS; CURRIE, 2004; TAERUM et al., 2007), pelo qual apresenta quimiotaxia positiva (atração pelo fungo simbionte) (GERARDO et al., 2006). Currie et al. (1999b) e Currie (2001a) mencionam que todas as evidências indicam que *Escovopsis* sp. só se encontra em colônias de formigas Attini e que, sua transmissão é horizontal através de pequenos artrópodes que ocasionalmente visitam os ninhos dessas formigas mas não há até o momento comprovação deste fato. Até o momento ainda é desconhecido o reservatório natural deste fungo.

O dano causado por esse fungo, segundo estes autores, consiste na redução da biomassa do jardim de fungos, com consequente redução da produção de pupas, larvas e operárias (CURRIE, 2001b). Segundo Reynolds e Currie (2004), *Escovopsis* sp. é um fungo micófago (necrotrófico) e secreta compostos que degradam a parede celular do fungo simbionte e se alimenta dos nutrientes que ficam disponíveis, mas tal fato ainda não foi devidamente comprovado, não havendo identificação das enzimas ou dos compostos secretados pelo fungo.

1.7.4 Bactérias

Assim como outros micro-organismos, as bactérias podem ser isoladas dos ninhos e do exoesqueleto das formigas.

(a) *No jardim*

Craven et al. (1970), encontraram bactérias no material de descarte em ninhos de *Acromyrmex octospinosus*, assim como, maior quantidade de bactérias no material descartado dos formigueiros de *Atta cephalotes*. Kermarrec et al. (1986), em seus estudos realizados com ninhos de *Atta laevigata*, confirmaram a presença de bactérias no jardim de fungos e identificaram pelo menos seis espécies de *Bacillus*. Além disso, Ribeiro (2000), trabalhando com ninhos de *Atta sexdens*, isolou dezoito espécies de bactérias compreendendo os gêneros *Bacillus*, *Klebsiella*, *Staphylococcus*, *Citrobacter*, *Leclercia*, *Brevibacillus*, *Paenibacillus* e *Pantoea*. Algumas dessas bactérias, talvez sejam mutualistas, secretando compostos que ajudam na degradação e preparação do substrato ou, sejam simples parasitas da associação formiga-fungo, retirando parte da energia do sistema

(PAGNOCCA et al., 2011). No entanto, a verdadeira função desses micro-organismos ainda é pouco conhecida. Sabe-se que bactérias do gênero *Burkholderia* secretam compostos que inibem a germinação de esporos de *Escovopsis* sp. e do fungo entomopatogênico *Metarhizium anisopliae* (SANTOS et al., 2004).

Mueller et al. (2008) isolaram do jardim e do pellet da cavidade infrabucal das rainhas, duas actinobactérias dos gêneros *Mycobacterium* e *Microbacterium*. Os autores propõem que elas poderiam atuar na proteção dos jardins de fungos contra micro-organismos indesejáveis. Também, mencionam que estes dois gêneros são componentes das comunidades de bactérias do solo e do material vegetal.

A atividade bioquímica de micro-organismos isolados de ninhos de *Atta sexdens* foi analisada e constatou-se que tanto bactérias como leveduras apresentaram atividade proteolítica, amilolítica e pectinolítica (BACCI et al., 1995; CARREIRO et al., 1997; CARREIRO, 2000; RIBEIRO, 2000; SILVA et al., 2006; MENDES et al., 2012). Segundo Bacci et al. (1995), a pequena porcentagem de bactérias degradadoras de celulose encontrada, poderia representar uma seleção de bactérias celulolíticas que, juntamente com o fungo simbionte, o qual também degrada celulose, atuariam sinergisticamente.

Trabalhando com jardim de fungo de *A. cephalotes*, Pinto-Tomás et al. (2009) mostrou que as bactérias podem fixar nitrogênio e entre elas, aquelas do gênero *Klebsiella* pareceram ser os mais importantes fixadores. Mais recentemente, Suen et al. (2011) encontraram que existe uma comunidade de bactérias no jardim de fungo com uma alta capacidade de degradação de lignocelulose em jardins de fungos de *A. colombica*.

(b) *Sobre o exoesqueleto da formiga*

Sempre foi observado sobre o corpo de algumas espécies de *Acromyrmex* a ocorrência de partes esbranquiçadas, a qual foi considerada inicialmente como sendo uma secreção de hidrocarbonetos. Entretanto, Currie et al. (1999b) encontraram que essa manchas brancas são na realidade uma bactéria filamentosa, ou seja, trata-se de uma actinobactéria. Este tipo de bactéria tem a particularidade de secretar metabólitos secundários entre os quais se encontram aqueles com propriedades antifúngicas, que neste caso tinha uma atividade específica contra o parasita *Escovopsis* sp. Essa bactéria ocorre sobre o corpo de operárias em cavidades denominadas “fóveas”, as quais têm conexão mediante poros com glândulas formadas por unidades bicelulares (CURRIE et al., 2006). Os autores sugerem que tais

glândulas secretam compostos através dos quais as bactérias se nutrem. Inicialmente considerada como pertencente ao gênero *Streptomyces* (CURRIE et al., 1999a), esta bactéria foi posteriormente classificada no gênero *Pseudonocardia* (CURRIE et al., 1999a; CURRIE et al., 2003; CAFARO; CURRIE, 2005). Uma combinação de provas moleculares indica que a interação formiga-*Pseudonocardia* teria coevoluído junto ao fungo simbionte e ao parasita *Escovopsis* sp. (CAFARO; CURRIE, 2005; CURRIE et al., 2006; CALDERA et al., 2009). Oh et al. (2008), num recente trabalho, isolaram a bactéria *Pseudonocardia* sp. da cutícula de *Apterostigma dentigerum* e comprovaram que ela inibe o crescimento de *Escovopsis* (micoparasita) proveniente da mesma colônia. O composto inibidor do fungo parasita foi identificado como dentigerumicina (OH et al., 2009). Porém, os mesmos autores demonstraram que dentigerumicina também é ativa contra outros micro-organismos, incluindo várias linhagens de *Candida albicans* (OH et al., 2009), revelando que o composto não possui especificidade para *Escovopsis* sp.

Ribeiro (2000) isolou bactérias filamentosas da cutícula de operárias de *Atta sexdens*, identificando-as como *Streptomyces setonii*. Recentemente, Haeder et al. (2009) isolaram bactérias dos gêneros *Pseudonocardia*, *Streptomyces* e *Dermacoccus* de pequenas porções do jardim e do corpo de outras espécies de formigas, a saber: *A. octospinosus*, *A. volcanus* e *A. echinatior*, e encontraram que *Streptomyces* sp. foi a actinobactéria que secretou um composto identificado como candicidina o qual inibiu o crescimento do fungo *Escovopsis* sp. Assim, fica claro que vários actinomicetos podem ser isolados das cutículas das operárias (KOST et al., 2007; HAEDER et al., 2009). Em especial, foi demonstrado através de análises filogenéticas, que muitas *Pseudonocardia* spp. isoladas de diferentes formigas Attini são semelhantes a outras linhagens comumente encontradas no solo (MUELLER et al., 2008). Em conjunto, essas evidências sugerem que *Pseudonocardia* sp. talvez não apresente uma história evolutiva tão antiga como foi sugerido anteriormente.

Com esta breve revisão, espera-se ter mostrado, ao menos parcialmente, o estado-da-arte no que diz respeito ao conhecimento da microbiota comumente encontrada em associação com as formigas da tribo Attini. Numerosos outros estudos abordando a filogenia das formigas e de seus simbiontes estão disponíveis na literatura, bem como muitos outros estudos relacionados com a biologia dessas formigas e sua interação com o homem e outros seres.

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3 SOBRE OS CAPÍTULOS

O projeto inicial e principal foi realizar um estudo preliminar da diversidade de leveduras provenientes de ninhos de quatro espécies de formigas cortadeiras (Attini derivadas) de uma região da província de Santa Fé, Argentina. Das quatro espécies de formigas incialmente previstas, a saber: *Acromyrmex heyeri*, *Acromyrmex lobicornis*, *Acromyrmex lundii* e *Atta vollenweideri*, apenas desta última não foi possível isolar leveduras dos jardins de fungo nem do depósito de descarte. Portanto, concentramos nosso trabalho nas três espécies de *Acromyrmex*. Entretanto, trabalhos e estudos de campo nos permitiram ampliar o projeto inicial, pois outras ocorrências interessantes foram verificadas, inclusive com outras espécies de formigas cultivadoras de fungos pertencentes ao grupo das Attini basais, mas desta vez, provenientes do Campus da UNESP, Rio Claro, Brasil.

Para abrigar toda a diversidade de nossos estudos, julgamos conveniente apresentar os dados em duas partes, cada uma delas constituída por capítulos. A primeira parte contém cinco capítulos e está relacionada com as formigas cortadeiras da Argentina e o Capítulo 1 trata da descrição dos ninhos das formigas com as quais trabalhamos. Apesar de, aparentemente, não ter uma relação direta com a Microbiologia, nossa intenção em incluí-lo foi conhecer detalhadamente os hábitos e os ninhos das espécies estudadas, para mostrar que os formigueiros devem ser considerados como um único organismo, onde vários sistemas biológicos se integram. Assim, ao lado do interesse puramente microbiológico, está também a necessidade de observar o sistema como um todo, integrado, pois consideramos que um sistema biológico, seja qual for, não pode ser totalmente explicado ou compreendido apenas em partes. Com isso queremos dizer que tudo o que acontece no interior do ninho (jardim de fungos, formigas, depósitos de rejeitos, micro-organismos do jardim, micro-organismos e compostos do solo, etc.) está relacionado com o exterior (condições ambientais, vegetação, constante interação das formigas com outros micro-organismos do entorno) constituindo um sistema intimamente inter-relacionado.

O Capítulo 2 é uma observação de campo que tivemos a felicidade de registrar e que decidimos apresentar porque a consideramos interessante e praticamente inédita; ainda, a observação amplia um pouco mais o conhecimento sobre o tipo de forrageamento de uma das espécies de formigas cortadeiras, *Acromyrmex lobicornis*. Os Capítulos 3, 4 e 5 descrevem os resultados do isolamento e identificação da comunidade de leveduras. O Capítulo 3 é um estudo da diversidade de leveduras encontradas nos jardins e nos depósitos

de descarte dos ninhos das três formigas cortadeiras - *Ac. heyeri*, *Ac. lobicornis* e *Ac. lundii* - ao longo das quatro estações do ano, as quais, nesta região, têm marcadas características ambientais. Já os Capítulos 4 e 5 estão relacionados à descrição de duas espécies novas de leveduras dos gêneros *Wickerhamomyces* e *Rhodosporidium*, respectivamente. Ambas as espécies foram isoladas de um ninho de *Ac. lundii*, proveniente do jardim de fungo (a primeira) e do depósito de descarte (a segunda). A segunda parte, relacionada com formigas basais do Brasil, está formada por dois capítulos. O Capítulo 1 trata da observação, no fungo cultivado por *Mycocepurus smithii*, de estruturas que denominamos de "gongylidia-like structures" devido a sua semelhança com estruturas unicamente encontradas nos fungos das formigas Attini derivadas, os "gongilideos". Estas estruturas sempre foram consideradas como uma característica evolutiva única do fungo cultivado pelas Attini superiores, mas a nossa observação da ocorrência de estruturas similares em Attini basais, de certa forma, está modificando esse conceito, mantido desde os estudos iniciais nessa área, ou seja, no século XIX.

Finalmente, o Capítulo 2 desta segunda parte mostra a descrição de uma espécie nova de fungo micoparasita pertencente ao gênero *Escovopsis* isolado do jardim de fungo de *Mycocepurus goeldii*. Atualmente, só existe duas espécies descritas deste gênero: *E. weberi* e *E. aspergilloides. E. weberi* foi isolado de jardins de fungo de *Atta* sp. e *E. aspergilloides* foi isolado de jardins de fungos de *Trachymyrmex ruthae*. Ambos os gêneros de formigas pertencem ao grupo das formigas com agricultura derivada. Além de se tratar de uma provável espécie nova no gênero, este trabalho será a primeira descrição de um fungo deste gênero em formigas basais.

Cada um dos capítulos está redigido no formato das revistas para os quais pretendemos submetê-los em breve.

No Apêndice apresentamos um artigo do qual participamos durante o período em que cursamos o doutorado.

4 PRIMEIRA PARTE

FORMIGAS ATTINI DA ARGENTINA

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4.1 CAPÍTULO I

Architecture of adult nests of three *Acromyrmex* species from Santurce, Santa Fé province, Argentina

Target journal: Jounal of Natural History

Title: Architecture of adult nests of three *Acromyrmex* species from Santurce, Santa Fé province, Argentina

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Architecture of adult nests of three *Acromyrmex* species from Santurce, Santa Fé province, Argentina

Abstract

Within the class Insecta the individuals of Hymenoptera and Isoptera orders are considered the most diverse and complex nest builders. Tribe Attini (Hymenoptera: Formicidae) ant nests are built as mounds on the ground, on trees, in subterranean galleries and some of them may be covered with loose soil, sticks or thatch. Internally, they could have few or several chambers. The nests are important for the protection of the brood, the queen and the food source, and inside it requires controlled environmental conditions (temperature, humidity, and air circulation). Considering that *Acromyrmex* ants may have an important role in defoliation of crops, knowing the architecture of the nests may be useful when methods for the control need to be applied. In this work we present a description of external and internal nest architecture of 30 nests of three species of *Acromyrmex* ants (*A. heyeri*, *A. lobicornis* and *A. lundii*) from the department of Santa Fé, Argentina. Our goal is that this detailed description serves as a guide for easily recognition in the field and the internal structure assist in studies of control strategies.

Keywords: Attini, *A. heyeri*, *A. lobicornis*, *A. lundii*, nest-building, leaf-cutting ants

Introduction

Eickwort (1981) defined a nest as " a structure in which eggs are deposited and food for the resulting nymphs or larvae is brought from outside the structure by the parent(s)"; he proposed this definition taking into account the most diverse nest builders of the Hymenoptera order, from which the tribe Attini (Formicideae: Myrmicinae) is member.

Atta and *Acromyrmex* are two well-known genera of fungus-growing Attini ants as they cut fresh plant material as leaves, flowers, fruit and seeds, which are utilized as substrate for the fungus they cultivate (Möller 1893, Wheeler 1907, Weber 1972). As a result of this mixture of cultivated fungus and substrate, the ants build a sponge-like delicate structure known as "fungus garden", which are found in chambers of variable size (Wheeler 1907, Bruch 1922, Mac Donagh 1937). The fungus (Basidiomycota: Agaricales) is the sole

food source for the larvae, the queen and, in some cases, for the workers (Weber 1972, Quinlan and Cherrett 1979, Bass and Cherrett 1995). The foraged material may be collected from dicot or monocot plants depending on the ant species. *A. heyeri* strictly forage on monocot plants (grass-cutting ants), while *A. lundii* forage on dicot plant, and *A. lobicornis* cut both types (Bonetto 1959, Gonçalves 1961, Franzel and Farji-Brener 2000).

For optimal development and growth, the fungus needs specific environmental condition such as temperature, moisture concentration and composition of the air (Quinlan and Cherrett 1978, Powell and Stradling 1986, Kleideidam and Roces 2000, Keideidam et al. 2001, Bollazzi and Roces 2007). *A. vollenweideri* Forel builds subterranean nests with loose soil mound. An established nest may measure 8-10 m in diameter, 2.5-5 m in depth, having 2500- 3080 chambers, conical waste deposit, spherical chambers with fungus garden, superficial trails and colonies with 4-7 million of individuals (Hölldobler and Wilson 1990, Forti et al. 2011). *Acromyrmex* nests are smaller than the *Atta;* and both are built subterraneaneously, having a mound shape on the surface, which may have a cover of dry sticks and soil, or thatch (Bonetto 1959, Gonçalves 1961, Della Lucia and Moreira 1993). Some *Acromyrmex* nests consist of a single main chamber and small accessory chambers (*A. lundi, A. heyeri*), or may contain several irregular chambers with different sizes (*A. lobicornis*) (Bonetto 1959, Golçalves 1961). Leaf-cutting ants are known as pests because they attack several types of plants causing serious damage in the growth although there is little evidence that the majority of the taxa are of significant economic importance (Fowler et al. 1990). The three species of *Acromyrmex* studied in this work are considered pests in Argentina.

In this work we provide a detailed description of the external and internal structure of *A. heyeri, A. lobicornis* and *A. lundi* nests with the aim that serve to identify easily the nests in the field and the internal structure may be considered in the applications of control strategies.

Materials and methods

The field work was carried out in Santurce town (30º11’08.57” S; 61º11’07.87”W), Santa Fé province, Argentina during the four seasons of 2007/2008/2010/2011. Santurce is located in the Chaco phytogeographical province, Chaco domain. The climate is continental

with summer rain between 500-1200 mm and an average annual temperature of 20-23°C (Cabrera and Willink 1980). The predominant vegetation is xerophytic deciduous forest, with an herbaceous layer of grass (*Setaria, Digitaria, Trichloris*), cacti (*Opuntia quimilo*, *Cereus coryne*) and bromeliads (*Bromelia serra*, *Dyckia ferox*); there are also coconut trees, savannas and halophyte shrub steppes. Among the tree species are *Schinopsis*, *Aspidosperma, Zizyphus, Tabebuia* and several species of *Prosopis* (Cabrera and Willink 1980).

We studied the nest architecture of mature nests (>5 years) of *Acromyrmex heyeri* (n=10), *Acromyrmex lobicornis* (n=10) and *Acromyrmex lundii* (n=10). Ants were identified by the Gonçalves keys (1961) and Della Lucia (1993) and additional literature.

The nests of *A. heyeri*, *A. lobicornis* and *A. lundii* were identified by the different and particular structure of each one. We recorded the size mound (length, width, height, and depth) and waste deposit (length, width, and height), and measures of entrances, tunnels and chambers with fungus garden were taken whenever possible.

Results

Acromyrmex heyeri nest (n=10)

Nest.– The external structure of nests is variable depending on the environment and the season. A typical nest without surrounding vegetation had 4-6 entrance holes with 2-2.5 cm in diameter located at ground level (Fig. 1A). The nests presented the typical cover constituted by a mixture of dry sticks, and pieces of dry leaves, but in this particular observation they also had small pieces of cow dung (Fig. 1B). In the cover there were holes of ~2cm in diameter, which continued in the different underlayers, being the external layer wet, and the internal dry (Fig. 1C). The external dimensions were 1.23±0.11m long, 1.05±0.13m wide and 0.34±0.11m high.

The dimension of the internal chamber was 1 m high x 0.30 m wide, where the newest portion of the fungus garden was located below the layer above ground. The oldest part of the fungus garden was located below ground level (0.40-0.50 m in depth) (Fig. 1D). Surrounding the base of the central chamber there were 10-20 small and irregular chambers with variable size between 8-12cm high and 25-30cm wide) with small fungus garden and

some pupae. The main fungus garden was close but not touching the walls. There was a space of 6-8 cm between the garden and the nest cover (Fig. 1E).

Some nests surrounded by plants were higher (65-70 cm) and in this case the depth of the subterranean chamber was between 15-20 cm. The fungus garden was very close to the ground level. A nest was found in summer which had a small lateral chamber (9 cm in height and 12cm in width) at 10 cm depth without fungus garden but with ~30 pupae at different stages of development (Fig. 1F) found at a lateral small chamber.

During the winter the nests were silent and no signal of activity could be observed. The central and accessory chambers were empty without fungus garden or ants. A minor chamber with a few worker ants was found at 40 cm deep. The ants were assembled like a ball of ~ 6 cm in diameter in which major workers formed an external cover, and minor workers formed a second cover, both protecting a small piece of fungus garden and the queen. No pupae were observed in this case.

Waste deposit.– The waste deposit was external and distant 10 cm from the nest. The pile of waste deposit was 0.88±0.24 m long, 0.47±0.13 m wide, and 0.03±0.01 m high (Fig. 1A). Generally, the nests had a single waste deposit, although some had two (Fig. 1A). The main part of the dump was dry exhausted substrate (yellow color) (Fig. 1G).

Acromyrmex lobicornis nest (n=10)

Nest.– Externally, the mound of *A. lobicornis* nest was 1.78±0.42 m long, 1.38±0.75 m wide, 0.33±0.07 m high and 40-70 cm deep (Fig. 2A). All had a cover with holes in the surface (0.80-1.03 m wide, 1.15-1.3 m long, and 1-2 cm high) constituted of dry sticks of different size and mixed with soil (Fig. 2B). Internally, there were labyrinths of irregular tunnels (Fig. 2C-D). More than one hundred irregularly distributed chambers were observed with sizes between 5-9 cm long and and 3 - 8 cm wide (Fig. 2E-F). Seeds of *Prosopis* sp (Fabaceae: Mimosoideae) were observed in the tunnel walls of 8 out 10 nests (Fig. 2G) whose tunnels were covered with an exudates. In the nests of this area we did not observe any change in the general structure over the different seasons.

Waste deposit.– In this ant species the waste deposit is dark brown, shaped like a half moon (Fig. 2A) and containing exhausted substrate and dead ants (Fig. 2H). It was

1.34±0.5 m long, 0.72±0.31 m wide, and 0.03±0.01 m high, and they may be 40-50 cm away from the nest. Some of their tunnels have openings on the surface.

Acromyrmex lundii nest (n=10)

Nest.– The nests were built interlaced to roots of trees (Fig. 3A). The mound had 4-5 trails which led to the entrance hole (2 cm in diameter) located 1-1.5 m from the nest, and leading to it subterraneously. The particular characteristic of these forage trails is that they were built forming an open canal, allowing the grass to grow on top of as a ceiling thereby the trails remain hidden. There were also entrances located on the top of the mound close to the trunk of the tree (Fig. 3B), once they used the trunk and the branches as forage trails. In some cases, it was observed a buildup of dry sticks on the top of the mound. The mounds were 1.82±0.12 m long, 1.42±0.17 m wide, 0.36±0.08 m high and 40-60 cm in deep. Tree roots had been cleaned and the fungus garden was hanging from them. The main fungus garden was 35-40 cm high and 30-35 cm wide (Fig. 3C-D). In the bottom, close to the tree roots the ants built a mattress consisted of pieces of dry leaves and dry sticks (Fig. 3E). A variable number of 10-15 small chambers measuring 4-5 cm high and 6-9 cm wide were located surrounding the upper part of the main chamber.

Waste deposit.– The dump material was brownish yellow and deposited near the nest or approximately 60-70 cm away (Fig. 3F). The dimensions were 1.69±0.30 m long, 0.78±0.13 m wide, and 0.03±0.02 m high (Fig. 3A). No dead ants were found in this material.

Discussion

Considering that Attini ants encompass more than 290 species with variable habitats and nest populations (Brandão et al. 2011) there is a paucity of information regarding the nest structure and architecture for many species, some of them occurring in Argentina. Some nests may be subterranean, superficial or even a combination of both types. They can also be covered by plant material, soil fragments or thatch with a single uncovered opening as in *A. striatus* or surrounded with straw tube as in *A. fracticornis* (Bonetto 1959, Gonçalves 1961, Weber 1972, Della Lucia and Moreira 1993, Verza et al. 2007). Internally, they can contain a main chamber with other secondary chambers oval or circular shaped or totally irregular (Bonetto 1959, Gonçalves 1961).

On the other hand, the internal architecture was characteristic of each ant species, in some cases presented plasticity depending on the environmental conditions. The waste deposits of the three species of *Acromyrmex* studied, i.e, A. *lundii, A. lobicornis* and *A. heyeri* were external and with specific characteristics such as morphology, color, texture, and moisture concentration.

A. heyeri nests

The nests of *A. heyeri* showed different types of construction depending on the abundance of surrounding vegetation and environmental conditions such as direct sunlight, temperature and humidity. Bollazi and Roces (2008) studied the thatch of *A. heyeri* nests and they found that thermoregulation is important for the nest, a fact that could explain the different number of thatch layers according to surrounding environment where more layers mean more insulating.

The external waste deposit of the ten nests of *A. heyeri* studied were made up only by exhausted substrate. No dead ants were found inside or oustside the nests.

A. lobicornis nests

The tunnels of the top of *A. lobicornis* nests are totally irregular connecting together and leading to a hole in the surface of the mound. Likely, these tunnels are aimed to the control of temperature and air circulation, because many of them have direct connection with the chambers. This hypothesis is supported by the fact that in nests surrounded by abundant vegetation the number of tunnels are smaller than that from nests growing in open areas.

The holes in the surface of mound are partially covered with dry sticks which suggests that, likely, they functioned as air filter preventing entry of impurities from the air (wind). We observed workers of *A. lobicornis*, *A. lundi* and *A. vollenweideri* carrying *Prosopis* seeds into the nests such as observed by Milesi and Lopez de Casenave (2004) in *A. striatus* and *A. lobicornis* from Mendoza province, Argentina. This observation would suggest that they develop the myrmecocory (the dispersion of diaspores by ants attracted by elaiosomes, Rico-Gray and Oliveira, 2007) or they use the seeds as substrate for the fungus, but in 8 out 10 nests of *A. lobicornis* the seeds were embedded in the walls of the tunnels. However, myrmecocory is unlikely because the seeds of *Prosopis* do not have elaiosomes, and it is considered as non-mymecochorous plant (Milesi and Lopez de Casenave, 2004).

The consistency and appearance of wet waste material are characteristics of this ant species. Farji-Brener and Ghermandi (2000) found that the dump is wet and with high content of of organic matter, nitrogen content, and phosphorus when compared to the adjacent soil.

A. lundii nests

Nests of *A.lundii* are usually associated to tree roots (Bruch 1922, Bonetto 1959, Weber 1972) having fungus gardens hanging from them. One particularity is that the mattress of sticks and leaves built in the bottom of the chamber is probably related with the maintenance of temperature and humidity of the chamber. In laboratory nests of *A. lundii* maintained at 25°C and 70% humidity, the ants use a part of the plant material to completely cover the fungus garden (V. E. Masiulionis personal observation).

Further studies are necessary to know if there exists a relationship between the fungus garden of *A. lundi* and the rhizosplane-rhizosphere. The microbial population in the rhizosphere depends on the structure of the radicular system (Atlas and Bartha 2005). The root exudates may release compounds as aminoacids, cetoacids, vitamins, sugars, tannins, alkaloids and phospholipids that may stimulate the growth of microorganisms from the rhizoplane (Rovira 1969, Atlas and Bartha 2005). It has been studied that there are bacteria in the rhizosphere producing protective biofilm or antibiotics controlling the possible pathogenic microorganisms (Bais et al. 2004, Bais et al. 2006). Members of the microbiota associated to rhizosphere may secrete vitamins, aminoacids, auxins, cytokinins and gibberellins that promote the plant growth (Alvarez et al. 1995) and the fungus garden may benefit from this relationship.

The three nests described in this work present characteristic architecture to each ant species which makes easily recognizable in the field, namely, in general appearance of the mound. *A. lundii* commonly built the nests associated with tree roots, *A. heyeri* covers the nests with a tacht and the mature *A. lobicornis* mounds have approximately the same dimensions with low cover of sticks. There are some shared characteristics between the nests, such as the forage trials are superficial in *A. heyeri* and *A. lobirnis* but in *A. lundii* form as a channels being part of them subterraneous. *A. heyeri* and *A. lundii* present a main chamber while *A. lobicornis* presents several and irregular chambers. The feature that the three ant species shared is external waste deposit.

Acknowledgements

Special thanks are expressed to A. Iozia (the owner of the San Cayetano field) and G. J. Masiulionis for his assistance during the fieldwork. V. E. Masiulionis was sponsored by a scholarship from CAPES/PEC-PG. This work was supported by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

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Legends

Fig. 1. *A. heyeri* nest. A. External architecture of nest with two waste deposit. B. The constitution of thatch. C. Diverse layers of thatch with holes and tunnels. D. General view of main and accessory chamber. E. The top of main chamber. F. Chamber with pupae but without fungus. G. Details of dump.

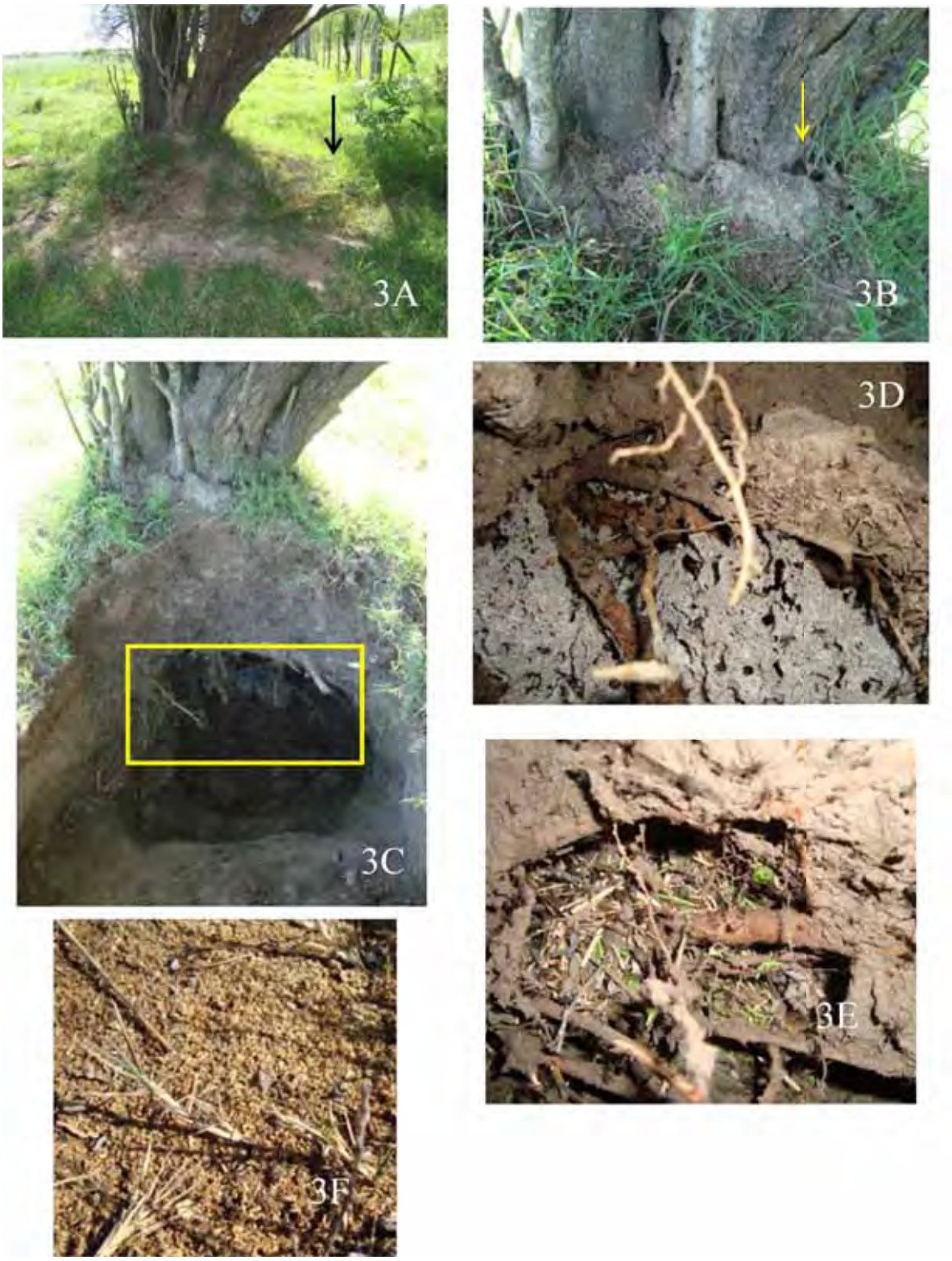
Fig. 2. *A. lobicornis* nest. A. External nest architecture. B. Detail of nest cover. C-D. Detail of the nest top showing the tunnel labyrinth. E. Details of the irregular chambers. F. Detail of tunnels that communicate with chambers and other tunnels. Also it show a seed pasted on the tunnel wall. G. Internal structure of the nest. H. Detail of the waste material.

Fig. 3. *A. lundii* nest. A. External nest architecture, the arrow shows the waste deposit. B. Arrow shows the holes for foraging between the tree trunk and mound. C. The main chamber and hanging fungus garden under the tree roots. D. Detail of fungus garden and the clean roots. E. Part of sticks and leaves mattress. F. Detail of waste material.

Fig. 1

Fig. 2

Fig. 3



4.2 CAPÍTULO II

Foraging of *Psilocybe* basidiocarps by the leaf-cutting ant *Acromyrmex lobicornis*

Target journal: Neotropical Entomology

Section: Scientific Note

Title: Foraging of *Psilocybe* basidiocarps by the leaf-cutting ant, *Acromyrmex lobicornis* (Emery) from Santa Fé, Argentina

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Running title: *Acromyrmex lobicornis* forages fungus

Abstract

An unusual diet selection of the leaf-cutting ant, *Acromyrmex lobicornis* (Emery), was observed in Santa Fé province, Argentina. Basidiocarps of *Psilocybe coprophila* (Bull) Kumm growing on cow dung were actively collected by workers (foragers). During this behavior the ants displayed typical signals of recognition and continuously recruited other foragers to the task. Parts of the fruiting body and even entire basidiocarps were being transported into the nest along with dicot and monocot plant material. No signal of basidiocarp rejection was observed. Until is let we know this unusual feature was recorded just one time before and may be part of an ancient behavior related to the origin of Attini ants' fungiculture.

Keywords: basidiocarps; *Deconica coprophila*; forage behavior; leaf-cutting ants; *Psilocybe coprophila*; coprophilous fungus

Ants in the genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae: Attini) are eusocial insects known as leaf-cutting ants because members of their foraging caste (workers) cut and carry fresh plant material, including leaves, flowers, fruit and seeds into the nest (Weber 1972). These activities are part of the foraging behavior which includes searching, selecting, cutting and transporting the plant matter (Wilson 1971, Wilson 1980). Foraging also involves several types of tactile and chemical communication among workers. When foraging workers find a suitable food resource, recruitment is triggered, and it involves different communication channels (Wilson 1971, Hölldobler 1999, Liefke et al 2001, Hölldobler & Wilson 2011). Plants to be cut are carefully selected according to physical parameters such as hardness or water content of leaves (Bowers & Porter 1981, Waller 1982, Nichols-Orians & Schultz 1989) as well as chemical characteristics such as toxins, terpenoids or antifungal compounds (Cherret 1972, Rockwood 1975, Rockwood 1976, Littledyke and Cherrett 1978, Hubbell et al 1984, Howard 1988). The preference for foraging on monocot, dicot or both groups of plants is related with the ant species (Fowler et al 1990, Franzel & Farji-Brener 2000, Lopes 2005). In the nest, the freshly cut material is extensively processed (Diniz & Bueno 2009) and cleaned with hygienic behaviors such as grooming and weeding (Currie & Stuart 2001), precedes the inoculation of the mutualist basidiomycete fungus *Leucoagaricus* (Lepiotaceae: Agaricales). This fungus serves as the source of food for the colony, mainly for the queen and broods (Weber 1972, Quinlan & Cherret 1979, Bass & Cherrett 1995) while the workers feed on plant sap, hyphae and staphylae as well (Littledyke & Cherrett 1976, Quinlan & Cherrett 1978, Quinlan & Cherrett 1979, Angeli-Papa & Eymé 1985, Bass & Cherret 1995, Murakami & Higashi 1997).

A. lobicornis is a leaf-cutting ant species distributed from subtropical areas in southern Brazil and Bolivia (23º S) through northern Patagonia, Argentina (44º S) (Farji-Brener & Ruggiero 1994). The nests can hold about 120 chambers which are built in part above the ground surface (25-50 cm) and partly underground (50-120 cm deep) (Bonetto 1959). The preference of *A. lobicornis* is to forage on dicot plants and sometimes collect monocots plants (Franzel & Farji-Brener 2000).

On 9 January 2010 at 10:35 am in Santurce (Santa Fé province, Argentina; 30° 11’ 16.14”S; 61° 10’ 24.35”W), foragers of an *Acromyrmex lobicornis* nest were observed to cut and carry basidiocarps of a coprophilous fungus. This region belongs to the Chaco phytogeographical province (Chaco domain) where the predominant vegetation is

xerophytic deciduous forest, with an herbaceous layer of grass, cacti and bromeliads (Cabrera & Willink 1980). We recorded these observations with videos and photographs using a Sony Cyber-Shot DSC-W120 camera. Recognition of the fungus and recruitment behavior of foragers were described. For fungal identification, fresh and dried fruit-bodies were examined with an Axio Scope A1 light microscope fitted with the digital camera ICc 3 (Carl Zeiss, Jena, Germany). A pure culture of the fungus was obtained by suspending a mature basidiocarp over an agar plate of potato dextrose agar (PDA) augmented with penicillin G and streptomycin sulphate (each at 200 mg l^{-1}). Germinating basidiospores were transferred to a fresh PDA plate and mycelium of a 7-d-old PDA culture was used for DNA extraction, PCR amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA as described in detail by Weber (2011). Sequence searches were performed in GenBank using the BLASTN function (Zhang et al 2000).

The observations of a foraging trail of *A. lobicornis* showed that one group of foragers was carrying pieces of dicot plants whilst another group was cutting and carrying to the nest basidiocarps (Fig. 1) which were growing on the surface of several pats of cow dung (supplementary online material 1). Cow dung pats were approximately 50 and 70 m away from the nest. During a period of 5 min, ten ants were observed entering their nest carrying entire basidiocarps or parts of them. The ants collected basidiocarps at different stages of development, including immature forms with the partial veil still covering the gill chamber as well as mature forms with exposed spore-producing gills. The mean length of eighteen basidiocarps (from the lower end of the stipe to the pileus) was 4.7±1.9 mm (mean±SD) being the extreme measure 0.8 mm and 0.2 mm. Four ways to make contact of recognition were displayed by all ants during their first approach, i.e. (1) with antennae; (2) with antennae and the first pair of legs; (3) with antennae, mandibles and the first pair of legs; and (4) with antennae and the first and second pairs of legs (supplementary material 1). The behavior of recognition of the fungi between workers appeared to be the same which is described for the recognition of the commonly collected material (Hölldobler 1971, Wilson 1971). The same repertoire of touches was exchanged between the ants carrying the fungus towards the nest and the others workers in the trail what seemed a behavior of recruitment (Wilson 1959, Wilson 1978, Jaffé & Howse 1979, Hölldobler & Wilson 1990). No signal of basidiocarp rejection from the nest was observed. On the basis of basidiocarps as well as mature basidiospores, identification of the coprophilous fungus as *Psilocybe coprophila* (Bull.) P. Kumm. [syn. *Deconica coprophila* (Bull.) P. Karst.] was unequivocal according to

keys of Watling and Gregory (1987) and Richardson & Watling (1997). The ITS rDNA sequence was deposited in GenBank under acession number JX235960 and confirmed *P. coprophila* (accession AJ519795) to be the closest available match, showing a sequence identity at 591 out of 595 nt overlap. Short-term tapes and photos of this behavior were taken and are available upon request.

Although *A. lobicornis* is known to cut a variety of dicot and monocot plants (Franzel & Farji-Brener 2000), fungi have been described just one time as being part of its collections. Indeed, to the best of our knowledge there is only one previous report of any leaf-cutting ant collecting fungal basidiocarps. This behavior was reported in *A. lundii* from Buenos Aires, Argentina, and the fungus in question was *Agrocybe cylindracea* fruiting on the surface of *Populus* bark (Lechner & Josens 2012). In their studies of *A. lundii*, Lechner & Josens (2012) confirmed that laboratory colonies incorporated basidiocarp material of *A. cylindracea* into fungus garden.

Witte and Maschwitz (2008) reported another ant species *Euprenolepis procera* (Emery) (Hymenoptera: Formicidae) from Southeast Asian rainforest which consume epigeic mushrooms. It is not known why *Acromyrmex* ants forage on *P. coprophila* or *A. cylindracea*. Some species of *Agrocybe* are poisonous mushrooms and some *Psylocybe* produce psilocybin, one of the major psychoactive alkaloid (Passie et al 2002). However, the mycelia of both fungi of the family Strophariaceae are rich in carbohydrates and proteins (Mueller et al 2001) and it may explain why they were being foraged.

The nutritional requirements of *Leucoagaricus* are unknown, and only a few information are available about the biology of *A. lobicornis* colonies and its fungus garden. It is accepted that fungus gardens of the Attini ants contain a diverse microbiota originated from soil and plant material (Pagnocca et al 2012), but except for some yeasts, there is no record of the presence of any larger basidiomycetes fungus other than *Leucoagaricus* itself.

Another interesting point to be raised in this work is related to origin of fungiculture. The ‘Consumption First’ model (Weber 1972) postulates that the fungus might initially be consumed directly by ants, a process which could lead to its cultivation and to mutualism once the ants have become capable of transmitting the fungus to offspring. More detailed field observations and analyses of the fungus garden contents might reveal why this ant species was foraging for these fungi when many other food resources were freely available

to them. Perhaps, during this field observation, *Acromyrmex* species were just recapitulating an ancient behavior which may given arise to the Attini fungiculture.

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Acknowledgments

We are grateful to A. Iozia (the owner of the San Cayetano field) and G. J. Masiulionis for his assistance during the fieldwork. V. E. Masiulionis was sponsored by a scholarship from CAPES/PEC-PG. This work was funded by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

Figure 1. Worker (forager) of *Acromyrmex lobicornis* carrying an immature basidiocarp of *Psilocybe coprophila*

Fig. 1

4.3 CAPÍTULO III

Yeast diversity from three leafcutter ant nests from a region of Santa Fé, Argentina

Target journal: Antonie van Leeuwenhoek

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A**bstract**

There exist few works about the yeast communities in nests of attine ants and those works involved nests of *Atta* species and one about nests of *Myrmicocrypta* species. We show a preliminary study of the yeast communities of nests of three *Acromyrmex* ant species (*Acromyrmex heyeri*, *Acromyrmex lobicornis* and *Acromyrmex lundii*) inhabitting in Santurce, Santa Fé province, Argentina. Using conventional plating and flotation techniques, we isolated from the fungus gardens and waste deposits, 465 yeast strains (16 genera and 39 species represented by 19 ascomycetous and 20 basidiomycetous yeasts) including a black yeast, *Exophiala spinifera.* Our results showed seasonal variability in the diversity of yeast species where the *A. heyeri* nests presented the most diversity in the fungus garden and waste deposits during winter and summer, being *Rhodotorula* sp. (fungus garden) and *Meyerozyma caribbica* (waste deposit) the dominant yeasts species. The diversity in the *A. lobicornis* nests was higher in spring in the fungus gardens and in winter in the waste deposit and the dominant species were *Trichosporon asahii* (fungus garden) and *Candida mucifera* (waste deposit). Finally, in the nests of *A. lundii*, we observed the main fungus garden diversity in winter and in the waste deposit in spring where the dominant species were *Galactomyces candidum* (fungus garden) and *Stephanoascus ciferrii* (waste deposit). Based on the analysis of the D1/D2 domains of the large subunit rDNA, we found probable new species of the genera *Wickerhamomyces*, *Yarrowia*, *Cryptococcus* and *Rhodosporidium*.

Keywords *Acromyrmex, A. heyeri*, *A. lobicornis*, *A. lundii*, Attini, fungi

Introduction

Yeast populations are commomly studied on several environment as terrestrial (Sláviková and Vadkertiová 2000;Yurkov et al. 2008, Yurkov et al. 2011, Yurkov et al. 2012), aquatic (Hagler and Mendoça-Hagler 1981; Sláviková and Vadkertiová 1992; Sláviková and Vadkertiová 1997), on fruit (Marksimova et al. 2009), on nectar flowers or phylloplane (Inácio et al. 2002; Fonseca and Inácio 2006, Nix-Stohr et al. 2008; Inácio et al. 2010). Yeasts are found associated with a great variety of insects such as beetles (Six 2003; Farrell et al. 2001; Suh et al. 2003), termites (Prillinger et al. 1996), and homoptera, bees and wasps (Ganter 2006).

Limited to the Neotropics (Hölldobler and Wilson 2011), the fungus-growing ants in the tribe Attini (Hymenoptera: Formicidae) maintain a mutualistic relationship (estimated in 50-60 my) with a basidiomycetous fungi of the family Lepiotaceae (Agaricales: Basidiomycota) (Chapela et al. 1994; Hinkle et al. 1994; Schultz and Brady 2008). The genera *Atta* and *Acromyrmex* cut several plant material (leaves, flowers, fruit, seeds) and carry them into the nest where they are processed to be used as substrate on which the fungus will be inoculated (Wheeler 1907; Weber 1972). With this mixture (fungus plus substrate), the ants build a sponge-like structure so- called fungus garden which is located within chambers in the nest (Weber 1972). The substrate is constantly renovated, and the exhausted material is discarded as waste deposit, which could be in certain chambers inside the nests, or outside the nests close to them (Forti et al. 2011). Besides the basidiomycetous fungus, other microorganisms such as bacteria, filamentous fungi, and yeasts are found within the ant nest, wherein they form a micro-ecosystem (Pagnocca et al. 2011). Yeasts found in ant nests have been less studied in comparison to other microorganisms. The first isolation of yeasts in nests of *Atta cephalotes* and *Acromyrmex octospinosus* was carried out by Craven et al. (1970) but he did not identify the species. Other isolations were made by Angelis et al. (1983) from nests of *Atta sexdens* and *Atta laevigata*, Carreiro et al. (1997) from nest of *Atta sexdens rubropilosa* while Rodrigues et al. (2009) isolated yeasts from nests of *Atta texana*. The new yeasts species *Blastobotrys attinorum* (Carreiro et al. 2004) and *Cryptococcus haglerorum* (Middelhoven et al. 2003) were isolated from nests of *Atta sexdens* while the recent new yeast, *Trichosporon chiarelli* (Pagnocca et al. 2010), was found in nests of *Myrmicocrypta camargoi*.

The Santa Fé province of Argentina, habors a great variety of leafcutter ants; among which are *Acromyrmex heyeri*, *Acromyrmex lobicornis* and *Acromyrmex lundii* (Bonetto 1959). The nest architecture of the three ant species are different from each other, having in common the characteristic of discarding their waste material outside and near the nests. These three ant species cut different plant material: *A. heyeri* is a grass-cutting ant (monocot), *A. lobicornis* cut monocot and dicot whereas *A. lundii* cut only dicot plants (Bonetto 1959).

As at the moment of writing this work, there are no studies about yeast communities in *Acromyrmex* nests, hence, it is impossible to compare communities; few studies of such existing were made with nests of *Atta* species. The goal of this work was to study the composition and diversity of the yeasts communities in the fungus garden and waste deposit of three *Acromyrmex* ant nest during the four seasons of the year.

Materials and methods

Study site and sample collection

The study was carried out in a field in Santurce town, Santa Fé, Argentina (30°11’02.48”S, 61°10’10.39”W) during winter 2009, spring 2009, summer 2010 and autum 2010. This area belongs to Chaco phytogeographical province (Chaco domain) characterized by an average annual temperature of 20-23°C continental climate and with summer rain between 500-1200 mm (Cabrera and Willink 1980). The vegetation is xerophytic deciduous forest, with an herbaceous layer of grass, cacti and bromeliads; there are also coconut trees, savannas and halophyte shrub steppes. Among the tree species are *Schinopsis*, *Aspidosperma*, *Zizyphus*, *Tabebuia* and several species of *Prosopis* (Cabrera and Willink 1980). Currently, this region is land management but has preserved areas.

Twelve ant nests of *A. heyeri*, *A. lobicornis* and *A. lundii* were sampled, namely, one nest for each season for each ant species. Samples were collected from fungus garden and waste deposit of each ant nest. For fungus garden samples, the nests were carefully opened just like internal chambers with the fungus. The samples of fungus were taken with sterile spoons and placed in sterile plastic containers with wet plaster (for the maintenance of humidity). The waste material was also taken with sterile spoons and placed in sterile polypropylene tubes. Both types of samples were kept on ice until reaching the laboratory.

Isolation of cultures

The community of yeast was taken from a 1g sample from each fungus garden and was homogenized in 9.0 ml sterile saline solution 0.85%; serial dilutions were made and spread 150 µl of dilution on selective media such as malt extract-yeast extract-soytone (MYP), yeast extract-malt extract-peptone-glucose-agar (YMA), Petri dish supplemented with 150 mg chloranfenicol l^{-1}, pH ~ 4 to suppress bacterial growth. Each sample was plated in duplicate. The dishes were incubated at 20°C for 4 days and examined daily for 15 days. To isolate the black yeasts, 150 µl of the sample was placed on Mycosel agar (BBL^{MT}) using the flotation technique (Iwatsu et al. 1981), where each sample was plated in 8 replicates. The dishes were examined daily for 20 days. The same procedure was applied for the treatment of the waste material samples. Colonies were differentiated into morphological types, counted when possible, and 4-5 representatives of every colony type were taken. The different strains were isolated from MYP, YMA, and Mycosel agar dish, purified, stored first in glucose/malt extract/ yeast extract/NaH_2PO_4 (GYMP) dish, and after in 15% glycerol plus GYMP broth at -80°C for long-term maintenance.

Identification of yeasts

For the identification of yeast community, the cultures were first grouped according to their morphological characteristics, subsequently, PCR-fingerprinting with microsatellite-specific oligonucleotides was used to group the cultures, following the method described by Sampaio et al. (2001). Strains with the same electrophoretic profiles were considered as conspecific and 1-2 strains were selected to sequence the rRNA region. DNA extraction was done according to Almeida *et al*. (2005). PCR amplification was made using the primer pairs which amplify the D1/D2 domains of LSU rRNAs, forward NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). The PCR products were purified by using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). The sequencing reaction was performed with the same primers used during the amplification and with ABI Prism® Big Dye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystem). The products of this last reaction were purified using 125 mM EDTA, 3M sodium acetate and ethanol. The resulting samples were placed in a 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited manually with the software BioEdit

Sequence Aligment Editor v. 7.0.5.3 (Hall, 1999).Nucleotide sequences were compared with sequences deposited in the NCBI-GenBank (www.ncbi.nih.gov).

Statistical analyses

The yeast species abundance and community structure were determined for fungus gardens and waste deposit of each ant species taking into account the four seasons. Probability of dominance was calculated as the ratio of number of samples where a species was most abundant over the total number of samples where this species was observed. To show if there exist relationships among the proportion of basidiomycetous and ascomycetous yeasts, yeast quantity, ant species (*A. heyeri*, *A. lobicornis*, *A. lundii*), environment (fungus garden, waste deposit) and season of the year (winter, spring, summer, autumn) statistical analyses such as Fisher's exact test and chi-square test were performed where possible. They were considered to be statistically significant at the level $p \leq 0.05$. The diversity of yeasts was studied using Simpson and Shannon index while Sorensen and Jaccard index were calculated to compare the shared species among the different seasons within nests of the same ant species as well as fungus gardens and waste deposits of the different three species.

Results

Number of yeasts

During this study we isolated a total of 465 yeast strains from the twelve ant nests sampled. This proportion is composed of 16 genera and 39 species being 19 ascomycetous and 20 basidiomycetous yeasts (Table 1). Analysis of the D1/D2 large subunit rDNA gene sequences of the studied yeasts suggested the presence of new species in the genera *Yarrowia*, *Cryptococcus* and *Rhodotorula* isolated from *A. heyeri* nests and, *Wickerhamomyces* and *Rhodosporidium* found in *A. lundii* nests (Table 1).

In the *A. heyeri* nests (n=4) were isolated 138 yeast strain (65 from fungus garden and 73 from waste deposit) being 7 genera, 19 species (7 ascomycete, 12 basidiomycete) (Table 2). The nests showed in the fungus gardens 96.92% dominance of basidiomycetes and in waste deposit 75.34% of ascomycetous yeasts. The dominant yeast species recorded

in the winter season, in the fungus garden was *Rhodotorula* sp (42%) and in the waste deposit was *M. caribbica* (31%)..

In the *A. lobicornis* nests (n=4) yeast strains comprising 10 genera, and 14 species (9 ascomycete, 5 basidiomycete) (28 from fungus garden and 71from waste deposit) were isolated (Table 3).The ascomycetous yeasts dominated in both environments, viz: 52.14% in fungus garden and 66.20% in waste deposits. During winter, the dominant species in fungus garden was *Trichosporon asahii* (63%) while *Candida mucifera* (54%) was more dominant in waste deposit during spring. No yeasts were isolated from the fungus garden of *A. lobicornis* during autumn, except for an endophytic fungus, *Retroconis fusiformis*.

In the *A. lundii* nests (n=4) it was isolated 228 yeast strains (79 from fungus garden and 149 from waste deposit) consisting of 13 genera, and 23 species (14 ascomycete and 9 basidiomycete) (Table 4). Similarly to the nests of *A. lobicornis*, the prevailence of ascomycetous yeasts was observed the in the fungus gardens (78.48%) and waste deposits (80.54%). The dominant yeasts in fungus garden was *Galactomyces candidum* (36%) in winter and *Stephanoascus ciferrii* (100%) in summer; whereas, in waste deposit the predominant yeast species were *Candida berthetii* (63%) in autumn, and *Meyerozyma guilliermondii* (20-25%) in the four seasons.

The Fisher' exact tests (Table 5) showed that the quantity of ascomycetous and basidiomycetous species in the nests of the three *Acromyrmex* depended or are associated with the seasons of the year. On the other hand, the chi-square tests showed that the quantities of these two groups of yeasts are also associated with ants (Table 6) and with the environment (Table 7).

Fisher's exact test and chi-square analysis showed that the number of yeast species are associated with (i) the season of the year and environment (fungus garden- waste deposit) which were isolated (Table 8), (ii) the seasons and the ant species (Table 9), and (iii) the environment (fungus garden- waste deposit) with the ant species (Table 10).

Diversity and similarity

The results of Shannon and Simpson index (Table 11-13) applied to the analysis of the diversity showed that in *A. heyeri* nest had the most diversity of yeasts species during winter (Shannon 1.47; Simpson 4.27) and summer (Shannon 1.36; Simpson 4.63). In the waste deposit was observed the most diversity in the winter (Shannon 1.78; Simpson 5.41) and summer (Shannon 1.03; Simpson 6). The lower yeast diversity was observed among fungus gardens and waste deposits in spring and autumn (Table 11). The Jaccard and Sorensen index showed that the four samples of fungus gardens (from different seasons) had no shared species between seasons (Table 12) whereas waste deposits shared species during winter-summer and winter-autumn.

The most diverse *A. lobicornis* fungus garden was in spring (Shannon 1.07; Simpson 4.2) and the least diverse in summer and winter (Table 13). For the waste deposits, the most diverse was in winter (Shannon 1.71; Simpson 4.75). In the fungus gardens and waste deposits were found shared yeast species between winter and spring in both environments (Table 14).

Concerning the fungus gardens of *A. lundii*, the most diverse was in winter (Shannon 1.91; Simpson 6.79) whereas the waste deposit was in spring (Shannon 1.85; Simpson 6.92) (Table 15). According to the index of similarity, the fungus gardens in winter and autumn shared yeast species, while in the waste deposits summer and autumn were the seasons with higher (or the highest) index (Table 16).

Finally, the similarity among the nests of the three ant species was also calculated, showing that the fungus gardens (Table 17) and the waste deposits (Table 18) of *A. lobicornis* and *A. lundii* shared yeast species.

Discussion

Our results showed that the number of basidiomycetous and ascomycetous yeasts, as well as the number of the yeasts strains, are associated with the ant species, season of year and the environment where they were isolated (fungus garden or waste deposit). Interestingly, the diversity of yeasts inside the fungus gardens and waste deposits of each ant species is particular to each one, as well as the shared yeasts between the fungus gardens and waste deposits of each ant species. Finally, the similarity index calculated between the total of fungus gardens and waste deposits of the three ant species showed that *A. lobicornis* and *A. lundii* are more similar, maybe because *A. lundii* cuts only dicot plant and *A. lobicornis* cuts mainly dicot, and in some cases monocot plants, while *A. heyeri* is a strictly grass-cutter ant.

Basidiomycetous yeasts (dominant during the four seasons) were isolated in the fungus gardens of *A. heyeri*, agreeing with Angelis et al. (1983) and Rodrigues et al. (2009) who found the same in the fungus garden of *Atta laevigata* and *Atta texana*, respectively. However, Carreiro et al. (1997) who, worked with lab nests of *Atta sexdens rubropilosa*, found a dominance of ascomycetous yeasts in the fungus garden and waste deposit occuring in a similar way to *A. lundii* nests, except in autumn, when prevalence of basidiomycetes was shown in the fungus garden. *A. lobicornis* nests had basidiomycetous yeasts in the fungus gardens during winter and spring, and ascomycetes during summer. The waste deposit showed dominance of ascomycetous yeasts only in winter, and ascomycetes were isolated during the other seasons.

For the first time, we isolated *Candida mucifera* found in the fungus garden of *A. heyeri*, and in the waste deposit of *A. lobicornis* and *A. lundii*; this species was only isolated from livers of anurans in the Amazon forest (Kocková-Kratochvílová and Sláviková 1988; Lachance et al. 2011). Moreover, *Clavispora opuntiae*, found in the fungus garden of *A. lobicornis* during summer, belongs to the group of cactophilic yeasts, which are considered cactus-specific (Starmer et al. 2006). *C. opuntiae* is found in rotting tissue and somatic tissue of succulent plants; however, it is known to be dispersed by vectors such as *Drosophila* spp. and the prickly pear moth, *Cactoblastis cactorum* (Starmer et al. 1988).

The black yeast species was isolated from waste deposit of *A. heyeri*, *Exophiala spinifera*, which is considered a pathogenic yeast that produces several mycotic infections such as cutaneous disease (phaeohyphomycosis) (Harris et al. 2009, Singh et al. 2012).

Another peculiarity was found *Trichosporon chiarelli,* in a fungus garden of *A. lundii*. This yeast species was recently described by Pagnocca et al. (2010) isolated from fungus gardens and waste deposits of the ant nest of *Myrmicocrypta camargoi* (Sosa-Calvo and Schultz 2010). Currently, *T. chiarellii* has only been found associated with nest of attine ants as Mendes et al. (2012) obtained isolations from nests of *A. heyeri* and *A. lundii* collected in the south of Brazil.

Carreiro et al. (1997) suggested that a possible origin of the yeasts in the fungus garden is related with the working ants that, during their foraging activity, are in contact with leaves, flowers and fruits which contain a microbiota on the surface, vectored by insects (Rosa et al. 1992). On the other hand, Pagnocca et al. (2008) when studying the pellets of *Atta laevigata*, only found three yeasts species *R. glutinis*, *Aureobasidium pullulans* and *Cryptococcus laurentii*, whereupon, the fungus gardens may have been colonized by other yeasts as proposed by Rodrigues et al. (2009) who suggested that some yeasts species may come from the soil or plant surface due to the presence of yeast genera such us *Cryptococcus*, *Candida*, *Rhodotorula* and *Trichosporon* (Botha 2006; Fonseca and Inácio 2006).

In relation to the yeasts isolated from waste deposit, Carreiro et al. (1997) concluded that the several arthropods associated with ant nests (Della Lucia et al. 1993) could disseminate the yeasts, however in the field nests the environmental factors such as rain, wind, or other animals could also be taken into account Rodrigues et al. (2009) suggested some explanations such as (i) the yeast population depended on the input of certain plants during different seasons, (ii) garden age or health, (iii) the yeast could inhabit the fungus gardens in some periods of the annual cultivation cycle, (iv) the population could vary as a function of simple sugar concentration. These explanations may be right, but the yeast community linked to the attine ant nests is part of a system which interacts not only with ants but also with other arthropods that inhabit the tunnels of nests (Della Lucia et al. 1993; Silva Araújo et al. 2011), several filamentous fungi (Rodrigues et al. 2005a; Rodrigues et al. 2005b; Rodrigues et al. 2008; Pagnocca et al. 2012), bacteria (Scott et al. 2010) and actinobacteria (Currie et al. 1999, Kost et al. 2007, Haeder et al. 2009, Barke et al. 2010) which combined should create a characteristic micro-environment. This set of microorganism (and arthropods, as vectors) could change during the seasons creating

adequate conditions for promoting the growth of some microorganisms, or inadequate conditions inhibiting the development of others.

This was a ground breaker work because the leafcutter ants from Argentina have been poorly studied. More studies are necessary to learn about the yeasts communities in the nests of different *Acromyrmex* ants.

Acknowledgments

The authors are grateful to R. E. Lecuona for laboratory facilities in IMyZA-INTA, Argentina during the isolation of yeasts. They also acknowledge A. Iozia (the owner of the San Cayetano field, Santurce) and G. J. Masiulionis for his assistance during the fieldwork. They also thank I. Dayo-Owoyemi for English review. V. E. Masiulionis was sponsored by a scholarship from CAPES/PEC-PG. This work was supported by the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil) and the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

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Table 1 List of total yeast species isolated from the fungus gardens and waste deposit of *A. heyeri*, *A. lobicornis* and *A. lundii* during the four seasons.

Yeast species	Ant species			Total	Closest relative	
	Ac. heyeri	*Ac. lobicornis*	*Ac. lundii*		%	Accession
Ascomycota						
Aureobasidium pullulans		4		4	99	FJ150917
Brettanomyces naardenensis		4	8	12	100	AY969108
Candida sp.			3	3	100	HM364288
Candida berthetii			45	45	99	GU246242
Candida catenulata			5	5	100	HQ860276
Candida davisiana		7	15	22	99	JQ277257
Candida mucifera	2	20	3	25	99	AJ508572
Clavispora opuntiae		8		8	100	JN544050
Exophiala spinifera	9			9	99	EU257702
Galactomyces candidum		3	13	16	100	JN974269
Galactomyces geotrichum		2	5	7	98	JN974282
Geotricum sp.			2	2	99	DQ912840
Meyerozyma caribbica	22			22	100	FN428948
Meyerorozyma guilliermondii	2	10	30	42	100	FJ515260
Stephanoascus ciferrii	15	1	18	34	99	DQ442681
Torulaspora delbrueckii			22	22	99	HE616749
Wickerhamomyces sp. nov.			7	7	96	JX049441
Yarrowia lipolytica	4		6	10	99	GU320002
Yarrowia sp. nov.	3			3	96	GU320002
Basidiomycota						
Cryptococcus sp.			6	6	99	GU585738
Cryptococcus albidosimilis	4			4	99	EU002808
Cryptococcus albidus	12			12	100	AF406906
Cryptococcus flavus	4			4	99	AF181540
Cryptococcus sp. nov.	4			4	96	FN428891
Cryptococcus laurentii	4	4	8	16	99	EF056218
Cryptococcus sp. nov.	3			3	97	FN428921
Cryptococcus liquefaciens	2			2	99	AF181515
Cryptococcus magnus			1	1	99	AF181851
Cryptococcus saitoi	6			6	100	AF181540
Cryptococcus sp. nov.	4			4	97	AF181540
Rhodosporidium santurcensis			4	4	97	JX192657
Rhodotorula mucilaginosa	7	7	4	18	99	FJ743623
Rhodotorula sp.	25			25	99	AF444745
Rhodotorula sp. nov.	6			6	97	AF444745
Trichosporon asahii		14		14	100	EU559350
Trichosporon chiarelli			6	6	100	EU030272
Trichosporon japonicum		2	4	6	100	AF308657
Trichosporon jirovecii		13	9	22	99	HM802131
Trichosporon laibackii			4	4	100	JN939468
Total	138	99	228			

Table 2 Quantity of yeast strains isolated from the fungus gardens and the waste deposits of *A. heyeri* nests (fungus garden/waste deposit) in each season of year.

Yeast species	Season			
	Winter	Spring	Summer	Autumn
Ascomycota				
Exophiala spinifera	0/9			
Candida mucifera		2/0		
Meyerozyma caribbica	0/19		0/2	0/1
M. guilliermondii		0/2		
Stephanoascus ciferrii	0/11		0/1	0/3
Yarrowia lipolytica	0/4			
Yarrowia sp. nov.	0/3			
Basidiomycota				
Cryptococcus albidosimilis	4/0			
Cryptococcus albidus	0/12			
Cryptococcus flavus			4/0	
Cryptococcus sp. nov.			4/0	
Cryptococcus laurentii	4/0			
Cryptococcus sp. nov.	3/0			
Cryptococcus liquefaciens			0/1	0/1
Cryptococcus saitoi			6/0	
Cryptococcus sp. nov.			4/0	
Rhodotorula mucilaginosa	4/2	1/0		
Rhodotorula sp.	11/1	7/1		5/0
Rhodotorula sp. nov.				6/0
Total environment	26/61	10/3	18/4	11/5
Total	87	13	22	16

Table 3 Quantity of yeast strains isolated from the fungus gardens and the waste deposits of *A. lobicronis* nests (fungus garden/waste deposit) in each season of year.

Yeast species	Season			
	Winter	Spring	Summer	Autumn
Ascomycota				
Aureobasidium pullulans		3/1		
Brettanomyces. naardenensis			0/4	
Candida davisiana	0/2	0/7		
Candida mucifera	0/2	0/18		
Clavispora opuntiae			8/0	
Galactomyces candidum	1/1	0/1		
Meyerozyma. guilliermondii	0/1			0/9
Stephanoascus ciferrii	0/1			
Basidiomycota				
Cryptococcus laurentii	0/2	0/2		
Rhodotorula mucilaginosa		0/4	2/1	
Trichosporon asahii	7/5	2/0		
Trichosporon japonicum		2/0		
Trichosporon jirovecii	3/10			
Total environment	11/24	7/33	10/5	0/9
Total	35	40	15	9

Table 4 Quantity of yeast strains isolated from the fungus gardens and the waste deposits of *A. lundii* nests (fungus garden/waste deposit) during the four season.

Yeast species	Season			
	Winter	Spring	Summer	Autumn
Ascomycota				
Brettanomyces naardenensis			0/3	0/5
Candida sp.				0/3
Candida berthetii			0/10	0/35
Candida catenulata	5/0			
Candida davisiana	3/0	3/9		
Candida mucifera	0/1	0/2		
Galactomyces candidum	11/2			
Galactomyces geotricum		5/0		
Geotricum sp.		2/0		
Meyerorozyma guilliermondii	3/7	0/8	0/5	1/6
Stephanoascus ciferrii	0/4	0/4	10/0	
Torulaspora delbruekii	0/2	12/0	0/2	0/6
Wickerhamomyces sp. nov.		7/0		
Yarrowia lipolytica	0/6			
Basidiomycota				
Criptococcus sp		0/6		
Cryptococcus laurentii	0/8			
Cryptpcoccus magnus	0/1			
Rhodosporidium sp. nov.		0/4		
Rhodotorula mucilaginosa	0/4			
Trichosporon chiarelli				6/0
Trichosporon japonicum	4/0			
Trichosporon jirovecii		0/6		3/0
Trichosporon laibackii	4/0			
Total environment	30/35	29/39	10/20	10/55
Total	65	68	30	65

Table 5 Distribution of Ascomycota (A) and Basidiomycota (B) among different four seasons and analysis of this distribution by Fisher's Exact Test.

Ant species	Division	#isolates	Winter		Spring		Summer		Autumn	
			# isolates Found	% of total	# isolates Found	% of total	# isolates Found	% of total	# isolates Found	% of total
A. heyeri[a]	A	57	46	80,70	4	7,02	3	5,26	4	7,02
	B	81	41	50,62	9	11,11	19	23,46	12	14,81
	Total	138	87	63,04	13	9,42	22	15,94	16	11,59
A.lobicornis[b]	A	59	8	13,56	30	50,85	12	20,34	9	15,25
	B	40	27	67,50	10	25,00	3	7,50	0	0,00
	Total	99	35	35,35	40	40,40	15	15,15	9	9,09
A.lundii[c]	A	182	44	24,18	52	28,57	30	16,48	56	30,77
	B	46	21	45,65	16	34,78	0	0,00	9	19,57
	Total	228	65	28,51	68	29,82	30	13,16	65	28,51

[a]Fisher's Exact Test p-value = 0.002153
[b]Fisher's Exact Test p-value = 1.569e-07
[c]Fisher's Exact Test p-value = 0.0004008

Table 6 Distribution of fungi isolated, according to the Division (Ascomycota (A)-Basidiomycota (B)), belonging to different ant species among the different seasons and analysis of this distribution by contingency tables and chi-square test.

Division	# isolates	Ant species								
		A. heyeri			*A. lobicornis*			*A. lundii*		
		Found	Expected	% of total	Found	Expected	% of total	Found	Expected	% of total
A	298	57	88,44	19,13	59	63,44	19,80	182	146,12	61,07
B	167	81	49,56	48,50	40	35,55	23,95	46	81,88	27,54

X-square= 56.5239, df = 2, p-value = 5.321e-13

Table 7 Distribution of yeasts isolated according to the division Ascomycota-Basidiomycota, among different environments (fungus garden and waste deposit) and analysis of this distribution by contingency tables and chi-square test.

Division	# of isolates	Fungus garden			Waste deposit		
		# of isolates		% of total	# of isolates		% of total
		Found	Expected		Found	Expected	
Ascomycota	298	83	114,71	27,85	215	183,28	72,15
Basidiomycota	167	96	64,28	57,49	71	102,71	42,51
Total	465	179			286		

X-square= 38.4508, df = 1, p-value = 5.615e-10

Table 8 Distribution of isolates belonging to different four ant species among the different environment (fungus garden-FG and waste deposit-WD) and analysis of this distribution by Fisher's Exact Test.

Ant species	Local	# of isolates	Winter		Spring		Summer		Autumn	
			# of isolates	% of total	# of isolates	% of total	# of isolates	% of total	# of isolates	% of total
A.heyeri[a]	FG	65	26	40	10	15,38	18	27,69	11	16,92
	WD	73	61	83,56	3	4,11	4	5,48	5	6,85
	Total	138	87	63,04	13	9,42	22	15,94	16	11,59
A.lobicornis[b]	FG	28	11	39,29	7	25,00	10	35,71	0	0,00
	WD	71	24	33,80	33	46,48	5	7,04	9	12,68
	Total	99	35	35,35	40	40,40	15	15,15	9	9,09
A.lundii[c]	FG	86	37	43,02	29	33,72	10	11,63	10	11,63
	WD	142	28	19,72	39	27,46	20	14,08	55	38,73
	Total	228	65	28,51	68	29,82	30	13,16	65	28,51

[a]Fisher's Exact Test p-value = 1.173e-06
[b]Fisher's Exact Test p-value = 0.000917
[c] Fisher's Exact Test p-value = 8.403e-06

Table 9 Distribution of yeasts isolated from the different ant species nests among the different season of the year and analysis of this distribution by contingency tables and chi-square test. (A*: A. heyeri*, B*: A.lobicornis*, C*: A. lundii*).

Ant species	# isolates	Winter			Spring			Summer			Autumn		
		# of isolates		% of total	# of isolates		% of total	# of isolates		% of total	# of isolates		% of total
		Found	Expected		Found	Expected		Found	Expected		Found	Expected	
A	138	87	55,49	63,04	13	35,9	9,42	22	19,88	15,94	16	26,71	11,59
B	99	35	39,81	35,35	40	25,76	40,40	15	14,26	15,15	9	19,16	9,09
C	228	65	91,69	28,51	68	59,33	29,82	30	32,85	13,16	65	44,13	28,51

X-square = 70.0518, df = 6, p-value = 3.99e-13

Table 10 Distribution of yeasts isolated from the different ant species nests (A*: A. heyeri*, B*: A.lobicornis*, C*: A. lundii*) among the different environment (fungus garden and waste deposit) and analysis of this distribution by contingency tables and chi-square test.

Ant species	# isolates	Fungus garden		% of total	Waste deposit		% of total
		# of isolates			# of isolates		
		Found	Expected		Found	Expected	
A	138	65	53,12	47,10	73	84,88	52,90
B	99	28	38,11	28,28	71	60,89	71,72
C	228	86	87,77	37,72	142	140,23	62,28
Total	465	179		38,49	286		61,51

X-square= 8.736, df = 2, p-value = 0.01268

Table 11 Shannon and Simpson index applied to yeast communities from fungus garden (a) and waste deposit (b) of *A. heyeri* nests.

(a)

Season	Shannon	Simpson
Winter	1.47	4.27
Spring	0.8	2.04
Summer	1.36	4.63
Autumn	0.68	2.2

(b)

Season	Shannon	Simpson
Winter	1.78	5.41
Spring	0.63	3
Summer	1.03	6
Autumn	0.95	3.33

Table 12 Jaccard and Sorensen index calculated for yeast communities found in the fungus garden (a) and the waste deposit (b) during the four season of year in nests of *A. heyeri*.

(a)

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0	0
1	3	-	-
1	4	-	-
2	3	0	0
2	4	0	0
3	4	-	-

NOTE: 1: winter; 2: spring; 3: Summer; 4: autumn

(b)

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0.111	0.2
1	3	0.222	0.364
1	4	0.222	0.364
2	3	0	0
2	4	0	0
3	4	1	1

NOTE: 1: winter; 2: spring; 3: Summer; 4: autumn

Table 13 Shannon and Simpson index applied to yeast communities from fungus garden (a) and waste deposit (b) of *A. lobicornis* nests.

(a)

Season	Shannon	Simpson
Winter	0.85	2.29
Spring	1.07	4.2
Summer	0.5	1.55
Autumn	-	-

(b)

Season	Shannon	Simpson
Winter	1.71	4.75
Spring	1.29	2.91
Summer	0.5	1.66
Autumn	0	1

Table 14 Jaccard and Sorensen index calculated for yeast species found in the fungus garden (a) and the waste deposit (b) during the four season of year in nests of *A. lobicornis.*

(a)

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0.429	0.6
1	3	0.286	0.444
1	4	0	0
2	3	0.286	0.444
2	4	0	0
3	4	0	0

NOTE: 1: winter; 2: spring; 3: Summer; 4: autumn

(b)

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0.4	0.571
1	3	0	0
1	4	0.125	0.222
2	3	0.143	0.25
2	4	0	0
3	4	0	0

NOTE: 1: winter; 2: spring; 3: Summer; 4: autumn

Table 15 Shannon and Simpson index applied to yeast species isolated from fungus garden (a) and waste deposit (b) of *A. lundii* nests.

(a)

Season	Shannon	Simpson
Winter	1.91	6.79
Spring	1.74	4.01
Summer	0	1
Autumn	0.89	2.5

(b)

Season	Shannon	Simpson
Winter	1.75	6
Spring	1.85	6.92
Summer	1.2	3.22
Autumn	1.14	2.32

Table 16 Jaccard and Sorensen index calculated for yeast species found in the fungus garden (a) and the waste deposit (b) during the four season of year in nests of *A. lundii*.

(a)

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0.083	0.154
1	3	0	0
1	4	0.1	0.182
2	3	0	0
2	4	0	0
3	4	0	0

NOTE: 1: winter; 2: spring; 3: Summer; 4: autumn

(b)

1°Sample	2° Sample	Jaccard	Sorensen
1	2	0.167	0.286
1	3	0.222	0.364
1	4	0.182	0.308
2	3	0.1	0.182
2	4	0.083	0.154
3	4	0.667	0.8

NOTE: 1: winter; 2: spring; 3: Summer; 4: autumn

Table 17 Jaccard and Sorensen index comparing the yeasts communities isolated from the fungus gardens of three ant species.

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0.059	0.111
1	3	0.042	0.08
2	3	0.167	0.286

NOTE: 1: *A. heyeri*; 2: *A. lobicornis*; 3: *A. lundii*

Table 18 Jaccard and Sorensen índex comparing the yeast communities found in the waste deposits of three ant species.

1° Sample	2° Sample	Jaccard	Sorensen
1	2	0.167	0.286
1	3	0.19	0.32
2	3	0.529	0.692

NOTE: 1: *A. heyeri*; 2: *A. lobicornis*; 3: *A. lundii*

4.4 CAPÍTULO IV

Wickerhamomyces sp. nov., a new ascomycetous yeast isolated from the fungus garden of *Acromyrmex lundii* nest (Hymenoptera: Formicidae) from Santa Fé, Argentina

Target journal: International Journal of Systematic and Evolutionary Microbiology

Title: *Wickerhamomyces* sp. nov., a new ascomycetous yeast isolated from the fungus garden of *Acromyrmex lundii* nest (Hymenoptera: Formicidae) from Santa Fé, Argentina

Running title: New yeast isolated from fungus garden of ***Acromyrmex lundii***

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The GenBank submission number for the D1/D2 region of the LSU rRNA gene sequence of JLU025^T is ID1593413.

Summary

Seven yeast strains were isolated from the fungus garden of a field nest of *Acromyrmex lundii* located at Santurce, Santa Fe, Argentina. Sequencing of the D1/D2 domains of the large-subunit (LSU) rRNA and the internal transcribed spacer (ITS) regions, coupled with the absence of growth on methanol and *myo*-inositol, the negative diazonium blue B and urease reactions, and the assimilation of sucrose and D-xylose, and formation of hat-shaped ascospores indicated that the strains belong to an undescribed species in the genus *Wickerhamomyces.* Phylogenetic analysis of the sequences of the D1/D2 domains of the LSU rRNA showed that it belongs to the *Wickerhamomyces* clade, clustered with *W. subpelliculosus, W. anomalus,* and *W. ciferrii* being *W. subpelliculosus* the closest relative. The novel species differed from *W. subpelliculosus* in at least six physiological tests. The strains showed 96% of identity with *W. subpelliculosus* (Y-1683^{T}), 95% of identity with *W. anomalus* (Y-366^{NT}), and 95% of identity with *W. ciferrii* (Y-1031^{T}) in the D1/D2 domain of LSU rRNA. According to phenotypic and molecular results, the JLU025^{T} strain represents a novel species in the genus *Wickerhamomyces*, for which the name *Wickerhamomyces* sp. nov. is proposed, with the type strain JLU025^{T} (CBS 12756^{T}).

Introduction

The genus *Wickerhamomyces* was proposed by Kurtzman *et al.* (2008) after a phylogenetic analysis using a concatenated dataset of gene sequences from the small subunit (SSU) rRNA, the large subunit (LSU) rRNA, and the translation elongation factor-1α (EF-1α), and it consists of a group of species that were earlier nested in the polyphyletic group *Pichia* (Kurtzman & Robnett, 1998). According to this study, the *Wickerhamomyces* clade is the most divergent between the new clades *Barnettozyma* and *Lindnera* and there are no remarkable phenotypic properties that can be useful to define the genus. The main characteristics of the genus are the negative diazonium blue B and urease reactions, the absence of growth on methanol and *myo*-inositol, the assimilation of sucrose and D-xylose, and the formation of hat-shaped ascospores (Kurtzman *et al.*, 2011a). The twenty-six species currently accepted in the genus have been isolated from soil (van der Walt & Johannsen, 1975; Shin *et al*. 2011; Lintomg *et al*., 2012), plant material (Sláviková *el al*., 2007; Groenewald *et al*., 2011), tree exudates (Phaff *et al*., 1979; Spencer *et al*., 1996; de García *et al*., 2010), flowers of *Hibiscus* (Mushtaq *et al*., 2007), digestive tract of beetles (Pignal *et al*., 1988), insect frass (Kurtzman *et al*., 2011), larvae of dipterous (Rosa *et al*., 2009), natural fermentation of coffee cherries (Silva *et al*., 2000), and brined vegetables (Etchell & Bell, 1950). In this work, we describe a new species of *Wickerhamomyces* which was found inside a nest of *Acromyrmex lundii* ant (Hymenoptera: Formicidae). This ant species belongs to the tribe Attini which have a mutualistic relationship with basidiomycetous fungi in the order Agaricales. The ants cultivate this fungus as a food source for the colony and in turn the fungus is dispersed in nature by the ants and receives protection against competitors (Weber, 1972; Hölldobler & Wilson, 1990). To cultivate the fungus the ants employ different substrates (De Fine Licht & Boomsma, 2010) on which the fungus is inoculated building a delicate structure called "fungus garden" (Möller, 1893). *A. lundii* ants use pieces of leaves, flowers, and fruits of dicotyledonous plants as substrate for the fungal partner.

Materials and Methods

Seven strains were isolated from the fungus garden of the leaf-cutting ant *Acromyrmex lundii* (Hymenoptera: Formicidae). The field nest at Santurce, Santa Fe province, Argentina (30°10'52.40"S; 61°10'05.15"W) was collected in September, 2009.

Three samples of approximately 0.5g each of the fungus garden were homogenized in 5.4 ml sterile yeast extract/ malt extract/ peptone/ glucose broth (YMB) supplemented with 150 mg chloranfenicol l^{-1} and pH adjusted to 4 to suppress bacterial growth. One ml was inoculated in 9 ml of the same medium and after 4 days of incubation at 20 °C aliquots of 150 µl were spread on Sabouraud dextrose agar (Acumedia) supplemented with 150 mg chloranfenicol l^{-1} and pH adjusted to 4 to suppress bacterial growth. Colonies growing after 4 days of incubation were stored in GYMP medium (glucose/malt extract/yeast extract/NaH_2PO_4) at 6-8 ^oC and in 15% glycerol at -8 °C .

The strains were phenotypically characterized according to methods described in Kurtzman *et al*. (2011c). Genomic DNA was extracted following the protocol described by Sampaio *et al*. (2001). The ITS region was amplified with the primers forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and D1/D2 domain of the LSU rRNA gene was amplified using the pair primers forward NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). PCR amplification products were purified with illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and the sequencing reaction was performed with the same primers used during the amplification and with ABI Prism® Big Dye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystem). The products of sequencing reaction were purified using 125 mM EDTA, 3M sodium acetate and ethanol. The strands were sequenced in a 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited manually with the software BioEdit Sequence Aligment Editor v. 7.0.5.3 (Hall, 1999). The sequences were compared with those deposited in the GenBank.

The multiple sequence alignment was performed using MAFFT version 6 (Katoh & Toh, 2008) and the software package MEGA version 5.05 (Tamura *et al*., 2011) was used for the data analysis. The phylogenetic trees were constructed from the evolutionary distance data with Kimura´s two-parameter correction (Kimura, 1980) by the neighbor-joining method (Saitou & Nei, 1987). Bootstrap analysis (1000 replicates) was performed to assess the confidence limits of the branching (Felsenstein, 1985).

Results and Discussion

Comparison of results of molecular characterization from the D1/D2 domains of LSU rRNAs gene and of the ITS regions between the type strain and the six additional isolates confirmed that they are conspecific.

The phylogenetic analysis of the domains D1/D2 of the LSU rRNA nested *Wickerhamomyces* sp. nov, among four species (Fig. 1). Comparing the sequences of D1/D2 domain of LSU rRNA gene from JLU025^{T} and the closely related species *W. subpelliculosus* (Y-1683^{T}), *W. anomalus* (Y-366^{NT}), and *W. ciferrii* (Y-1031^{T}) we found 96% of identity (22 substitutions), 95% of identity (28 substitutions), and 95% (28 substitutions), respectively.

The novel species differ in at least six physiological tests (Table 1) from *W. subpelliculosus*, ten from *W. anomalus*, and eleven from *W. ciferrii*. *Wickerhamomyces* sp. nov. grows on melibiose, L-rhamnose, 5-keto-D-gluconate, and vitamin-free medium and at 37 °C while *W. subpelliculosus* does not.

Considering the analysis of the ITS region and D1/D2 domain of the LSU rRNA, and the morphological, biochemical and physiological properties, we inferred that strain JLU025^{T} and six other isolates from the same fungus garden of *Acromyrmex lundii* represent a novel anamorphic ascomycetous yeast species for which the name *Whickerhamomyces* sp. nov. is proposed. Probably the origin of the yeast strain was the plant material foraged by the ants.

Description of *Wickerhamomyces* sp. nov. Pagnocca & Masiulionis sp. nov.

Wickerhamomyces sp. nov. (holotype)

After 3 days growth on 5% malt extract agar at 25 C, cells are ellipsoidal and globose (2-3 x 5-7.5 µm), and ovoid, and occur singly or in pairs. Budding is multilateral. Pseudohyphae are formed. The streak culture is cream-coloured, butyrous, with a smooth surface and has entire margin. Asci are unconjugate and form one to four hat-shaped ascospores. Ascospores are abundant on cornmeal agar and acetate agar after 3-10 days at 20°C (Fig. 2).

The carbon compounds assimilated are: glucose, galactose, sucrose, maltose, cellobiose, melibiose, raffinose, melezitose, soluble starch (slow), D-xilose, L-rhamnose, ethanol, glycerol, erythritol (slow), D-mannitol, D-glucitol, methyl-α-D-glucoside, salicin, D-gluconate, citrate (weak), 5-keto-D-gluconato, xylitol, propane 1,2 diol (slow), and butane 2,3 diol (weak). While L-sorbose, lactose, inulin, L-arabinose, D-arabinose, D-ribose, D-glucosamine, N-acetyl-D-glucosamine, methanol, ribitol, galactitol, DL-lactate, succinate, *myo*-inositol, 2-keto-D-gluconate, saccharate, D-glucoronate, and L-arabinitol are not assimilated. Growth on nitrate, nitrite, cadaverine, L-lysine and vitamin-free is positive. No growth on creatinine, ethylamine, 50% glucose, 10% NaCl/5% glucose, 0.01 or 0.1% cycloheximide was observed. Production of starch-like compounds is negative. Fermentation is positive in glucose, sucrose and raffinose, but it is negative in galactose, maltose, lactose and trehalose. Urease and diazonium blue B are negative. Growth was observed at 25°C and 37 °C.

The type strain (JLU025^{T}) and the other six strains (JLU025D, JLU004A, JLU004B, JLU004D, JLU026A, JLU026B) were isolated from fungus garden of an ant nest of *Acromyrmex lundii* species (Hymenoptera: Formicidae) from Santurce, Santa Fé province, Argentina.

Acknowledgements

We are grateful to R. E. Lecuona for laboratory facilities in IMyZA-INTA, Argentina during the isolation of yeasts. We also acknowledge A. Iozia (the owner of the San Cayetano field, Santurce) and G. J. Masiulionis for his assistance during the fieldwork. We also thank Jonathan and C. B. Burgess for English review. V. E. Masiulionis was sponsored by a scholarship from CAPES/PEC-PG. Financial support by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

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Table 1. Physiological characteristics of JLU025^{T} (CBS 12756) that differentiate from closely related species.

Strains: 1, JLU025^{T} (CBS 12756); 2, *W. subpelliculosus* Y-1683^{T}; 3, *W. anomalus* Y-366^{NT}; 4, *W. ciferrii* Y-1031^{T}. Symbols: +, positive; -, negative; w, weak; s, positive but slow; v, variable; n, no data. ^{T}= type strain, ^{NT}= neotype strain.

Characteristics	1	2†	3†	4†
Carbon compounds:				
Galactose	+	v	v	+
Cellobiose	+	v	+	+/w
Melibiose	+	-	-	-
Raffinose	+	+	+	+
Melezitose	+	v	+	+
Soluble starch	s	v	+	+
D-Xylose	+	v	v	+/w
L-Arabinose	-	v	v	+/w
D-Arabinose	-	v	-	-
D-Ribose	-	v	v	+
L-Rhamnose	+	-	-	+/w
Erythritol	s	+	+	+
Ribitol	-	v	v	+
D-Gluconate	+	+	v	+
DL-Lactate	-	+	+	+
Succinate	-	+	+	+
Citrate	w	+	+	+
5-Keto-D-gluconate	+	-	-	-
Nitrogen compounds:				
Nitrate	+	+	+	+
Nitrite	+	+	+	n
Other tests:				
Vitamin free	+	-	+	+
Urease	-	-	-	-
DBB reaction	-	-	-	-
Growth at 37°C	+	v	v	w/-

†Data from Kurtzman *et al*. (2011b)

Figure legends

Fig. 1. Phylogenetic analysis of the D1/D2 domain of the LSU rRNA gene from JLU025^{T} and related species. Evolutionary distances data were calculated according to correction Kimura's two-parameter (Kimura, 1980) by the Neighbor-joining method using the MEGA version 5.0 software package. The numbers in brackets are GenBank accession numbers. Values of less than 50% not shown. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. *Wickerhamomyces* sp. nov. (JLU025^{T}). (a) Cells morphology after 3 days at 25°C on 5% malt extract agar; (b) Ascospores produced on cornmeal agar after 10 days at 20 °C.

Fig. 1

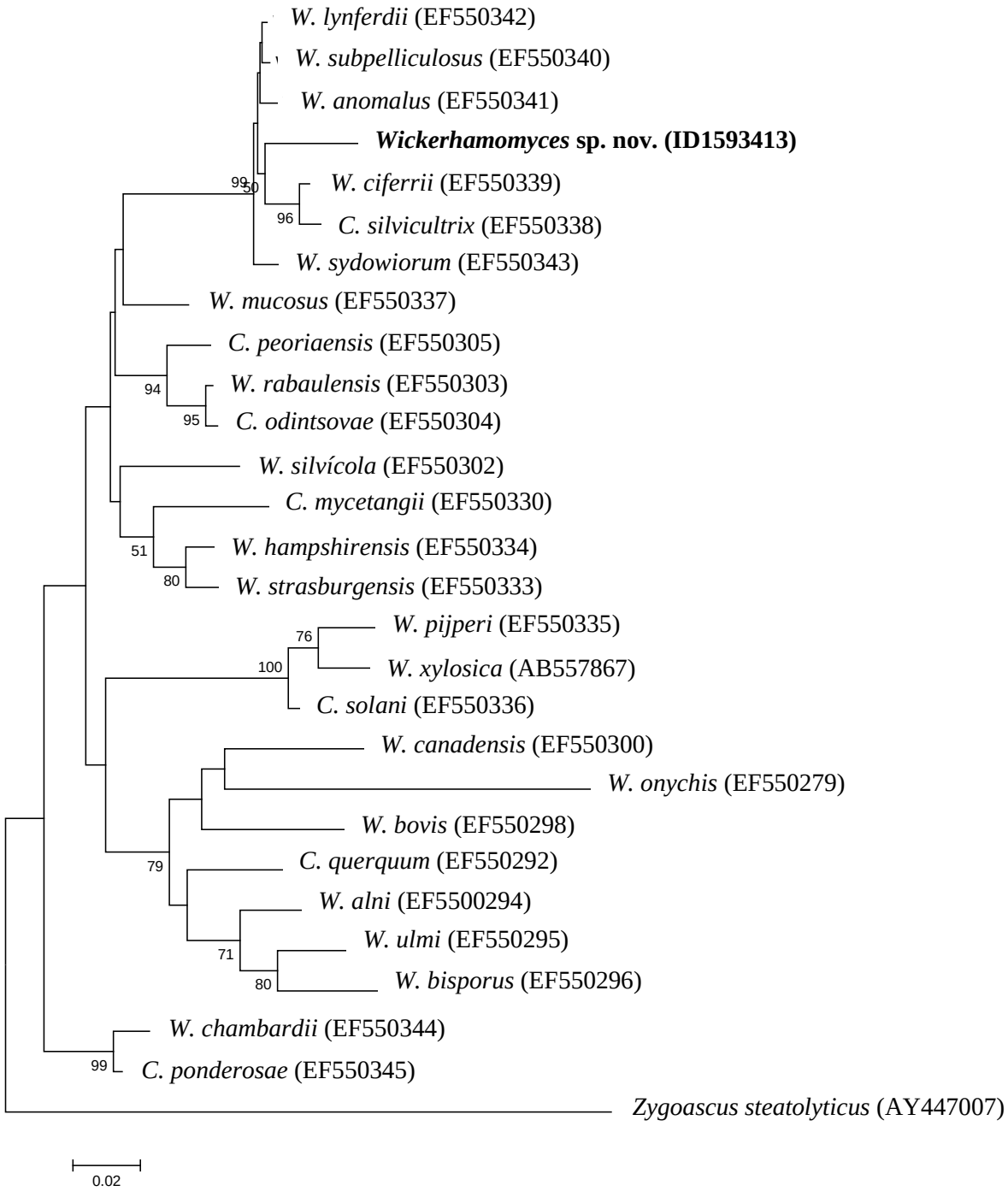
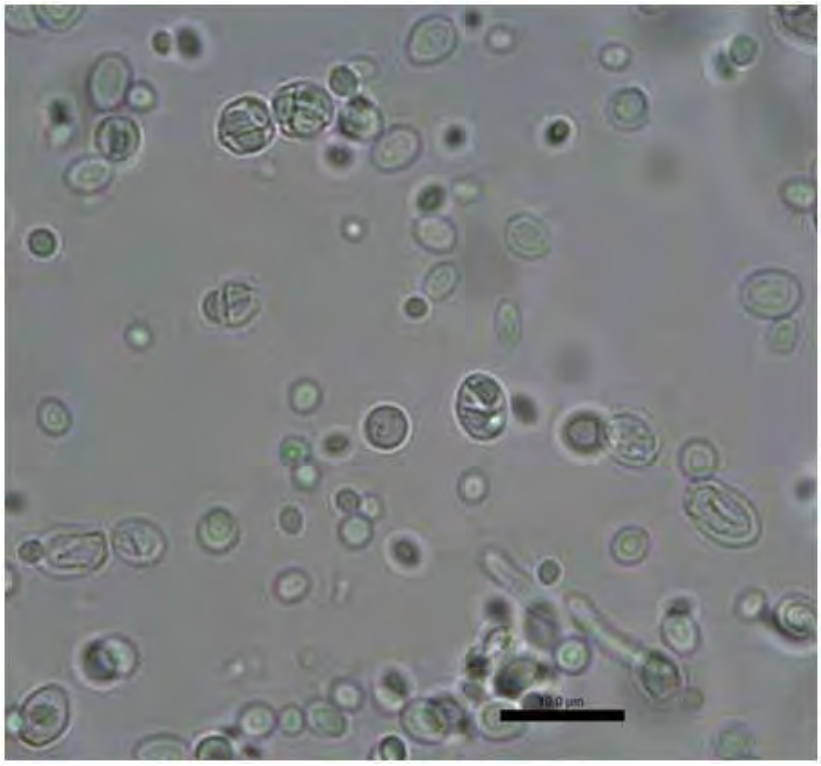

W. lynferdii (EF550342)
W. subpelliculosus (EF550340)
W. anomalus (EF550341)
Wickerhamomyces sp. nov. (ID1593413)
99
50
W. ciferrii (EF550339)
96
C. silvicultrix (EF550338)
W. sydowiorum (EF550343)
W. mucosus (EF550337)
C. peoriaensis (EF550305)
94
W. rabaulensis (EF550303)
95
C. odintsovae (EF550304)
W. silvícola (EF550302)
C. mycetangii (EF550330)
51
W. hampshirensis (EF550334)
80
W. strasburgensis (EF550333)
76
W. pijperi (EF550335)
100
W. xylosica (AB557867)
C. solani (EF550336)
W. canadensis (EF550300)
W. onychis (EF550279)
W. bovis (EF550298)
79
C. querquum (EF550292)
W. alni (EF5500294)
W. ulmi (EF550295)
71
80
W. bisporus (EF550296)
W. chambardii (EF550344)
99
C. ponderosae (EF550345)
Zygoascus steatolyticus (AY447007)
0.02

Fig. 2



4.5 CAPÍTULO V

Rhodosporidium santurcensis sp. nov., a new basidiomycetous yeast isolated from the waste deposit of *Acromyrmex lundii* nest (Hymenoptera: Formicidae) from Santa Fé, Argentina

Target journal: International Journal of Systematic and Evolutionary Microbiology

Title: *Rhodosporidium santurcensis* sp. nov., a new basidiomycetous yeast isolated from *Acromyrmex lundii* (Hymenoptera: Formicidae) nest from Santa Fé, Argentina

Running title**:** *Rhodosporidium santurcensis* sp. nov., associated to nest of Attini ant

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The GenBank submission number for the D1/D2 region of the LSU rRNA gene sequence of LLU043A^T is ID1590644.

Summary

Four novel yeast strains were isolated from the waste deposit of a *Acromyrmex lundii* (Hymenoptera: Formicidae) nest located in a field in Santurce town, Santa Fé province, Argentina. Based on D1/D2 domains of the large-subunit (LSU) rDNA and the internal transcribed spacer (ITS) regions, the four strains showed to be the same species. Characteristics such as the positive diazonium blue B and urease reactions, the absence of fermentation and the assimilation of L-sorbose, xylitol, L-arabinose and growth on vitamin-free medium indicated that theses strains belong to the genus *Rhodosporidium*. The phylogenetic analysis showed that the closely related species is *Rhodosporidium lusitaniae* with 95% identity in the ITS region and 97% identity in the D1/D2 domains of the LSU rRNA gene and in the physiological tests were found six differences. According to the phenotypic and molecular results, the four strains represent a novel species within the genus *Rhodosporidium*, for which the name *Rhodosporidium santurcensis* sp. nov. is proposed, with the type strain LLU043A^{T}.

Introduction

The genus *Rhodosporidium* (Banno, 1967) is located among the basidiomycetous yeasts which belong to the order Sporidiobolales (Sampaio *et al*. 2003). At present, this genus contain nine species described (Sampaio, 2011): *R. azoricum* Sampaio & Gadanho (2001), *R. babjevae* Golubev (1993), *R. diobovatum* Newell & I. L. Hunter (1970), *R. fluviale* Fell, Kurtzman, Tallman & Buck (1988), *R. kratochvilovae* Hamamoto, Sugiyama & Komagata (1988), *R. lusitaniae* Á. Fonseca & Sampaio (1992), *R. paludigenum* Fell &Statzell-Tallman (1980), *R. sphaerocarpum* Newell & Fell (1980) and *R. toruloides* Banno (1963) (Hamamoto *et al*., 2002; Sampaio, 2011). This genus can be found in terrestrial environments, associated to plants or soil (Fonseca & Sampaio, 1992; Sampaio, 1994; Gadanho *et al*. 2001; Sampaio *et al*., 2001; Hong *et al*. 2002), extreme environments such as hydrothermal vents and acidic mine water (Gadanho & Sampaio, 2005; Gadanho *et al*., 2006), coral reef (Newell & Hunter, 1970), seawater to marsh and mangrove swamp waters (Fell &Statzell-Tallman, 1980) or marine and estuarine environments (Gadanho *et al*., 2003; Libkind *et al*. 2003; Nagahama *et al*., 2001a; Almeida, 2005; Gadanho & Sampaio, 2005; Nagahama *et al*., 2006). The new species of *Rhodosporidium* was found in the waste deposit of the *Acromyrmex lundii* ant (tribe Attini: Formicidae). The Attini tribe is limited to the New World (Hölldobler & Wilson, 1990) and is known as "fungus growing ants" because they cultivate a basidiomycetous fungus (order Agaricales) as a food source for the colony (Weber, 1972; Mueller, 2002). Depending on the ant species, different materials are collected as substrates for the fungus growth (De Fine Licht & Boomsma, 2010). A particular group is known as "leaf-cutting ants" because cut plant material (leaves, flowers, fruits, seeds) which is carried out into the nest and is prepared for the inoculation of the fungus, forming a structure called "fungus garden" (Weber, 1972). The substrate is continually renovated and the exhausted substrate is discarded in a waste deposit which could be inside of the refuse chambers or outside of the nest, depending on the ant species (Forti *et al*., 2011; Hölldobler & Wilson, 2011). *Acromyrmex lundii* species from Santa Fé province (Argentina) is a leaf-cutting ant that disposes of the dump outside the nest (Bonetto, 1959).

Methods

Samples of waste deposit of a leaf-cutting ant *Acromyrmex lundii* (Hymenoptera: Formicidae) were collected in a field of Santurce, Santa Fe province, Argentina (30°10'52.40"S; 61°10'05.15"W) during spring season in September, 2009.

Four strains were isolated from a 1g sample of dump which was homogenized in 9.0 ml sterile saline solution 0.85%; serial dilutions were made and spread 150 µl of each dilution was spread in triplicate on malt extract/ yeast extract / soytone (MYP) Petri dish supplemented with 150

mg chloranfenicol l^{-1}, pH adjusted to 4. After inoculation for 6 days incubation at 20 °C the strains were isolated, purified, stored in glucose/malt extract/ yeast extract/NaH_2PO_4 (GYMP) tubes and in GYMP broth plus 15% glycerol at -80°C for long-term maintenance.

The identification of strains based on morphological, physiological and biochemical properties were carried out following to Kurtzman *et al*. (2011b). DNA extraction was done as described by Sampaio *et al*. (2001) and PCR amplification was made using the primer pairs forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for the ITS region and forward NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse NL4 (5'-GGTCCGTGTTTCAAGACGG-3') for D1/D2 domains of LSU rRNAs. The amplification products were purified by using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). The sequencing reaction was performed with the same primers used during the amplification and with ABI Prism® Big Dye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystem) and purified using 125 mM EDTA, 3M sodium acetate and ethanol. The resulting products were placed in a 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited manually with the software BioEdit Sequence Aligment Editor v. 7.0.5.3 (Hall, 1999).

Sequences of the type strrain of closely related taxa were downloaded from GenBank (www.ncbi.nlm.nih.gov). For multiple sequence alignment, MAFFT version 6 (Katoh & Toh, 2008) was used and the analysis of data was performed using the software package MEGA version 5.05 (Tamura *et al*., 2011). For the neighbor-joining method (Saitou & Nei, 1987), distances between the sequences were based on Kimura 2-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (1000 replicates) (Felsenstein, 1985).

Results and discussion

Molecular characterization of the type strain LLU043A^T and the other three strains through sequencing of the D1/D2 domains of LSU rRNAs and the ITS regions showed that the strains are conspecific.

Based on morphological and physiological tests, these strains belong to the genus *Rhodosporidium*. The comparative results of physiological and biochemical characters

between *R. santurcensis* and the two closely related species according to molecular studies can be seen in Table 1.

Consistent with the GenBank results, the comparison of type strains sequences with the sequences of the D1/D2 domain of the LSU rRNA from LLU043A strain showed 97% of identity with *Rhodosporidium lusitaniae* and *Rhodotorula colostri* and the ITS region showed 95% of identity with *R. lusitaniae*. Sequence analysis showed that all the four new strains differ from the *R. lusitaniae* type strain (AF070423) in the D1/D2 domain of the LSU rRNA by 13 substitutions and differs from *Rh. colostri* type strain (AY372177) by 16 substitutions. The ITS region of the *R. lusitaniae* type strain (AB073255) showed 28 substitutions. Phylogenetic analysis exhibited that the four strains belong to the *Rhodosporidium* clade where the closest relatives were *R. lusitaniae* and *Rh. colostri*. This clade is supported by 98% bootstrap values in neighbor-joining method.

Based on the analysis of D1/D2 domain of the LSU rRNA gene, ITS region, and the morphological, biochemical and physiological properties we concluded that these four strains represent a novel anamorphic basidiomycetous yeast species for which the name *Rhodosporidium santurcensis* sp. nov. is proposed.

Description of *Rhodosporidium santurcensis* Pagnocca & Masiulionis sp. nov.

Rhodosporidium santurcensis (san.tur.ce.en'sis N.L. nom. masc. adj. *santurcensis* referring to Santurce town in Santa Fé province, Argentina, where the nest of *A. lundii* was located.)

After 6 days at 20 °C, the cells are cylindrical to bacilliform, 2-3 x 6-9 µm, occur singly or in pairs and budding is polar (Fig. 2). The streak culture is pink colored, butyrous, shiny, smooth and with entire to slightly lobate margin. After 3 weeks no conjugation was observed.

Assimilation of carbon compounds is as follows: glucose, galactose, L-sorbose, trehalose, soluble starch (weak), D-xylose (weak), L-arabinose (slow), ethanol (weak), glycerol (slow), ribitol, galactitol, D-mannitol (weak), D-glucitol, gluconate, saccharate, xylitol (weak), L-arabinitol (weak) and propane 1,2 diol (slow). Sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, methanol, erythritol, methyl-α-D- glucoside, salicin, DL-lactate,

succinate, citrate, *myo*- inositol, 2-keto-D-gluconate, 5-keto-D-gluconate, D-glucuronate and butane 2,3 diol are not assimilated. Nitrate and nitrite (weak) are assimilated and growth on vitamin-free medium is positive. No growth occurs on cadaverine, creatinine, L-lysine, ethylamine, 50% glucose and 10% NaCl/5% glucose. Absence of fermentation and no growth occurs in the presence of 0.01 or 0.1% cycloheximide. Production of starch-like compounds is negative. Reactions with diazonium blue B and urease are positive. Growth was observed at 25 and 30 °C, but not at 35 °C.

Acknowledgements

We are grateful to R. E. Lecuona for using his laboratory in IMyZA-INTA, Argentina for the isolation of yeasts. We also thank A. Iozia (the owner of the San Cayetano field, Santurce) and G. J. Masiulionis for his assistance during the fieldwork. We also thank Jonathan and C. B. Bungess for English review. V. E. Masiulionis was sponsored by a scholarship from CAPES/PEC-PG. This study was supported by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

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Table 1. Physiological properties of LLU043A^T that differentiate from closely related species.

Strains: 1, *R. santurcensis* sp. nov. LLU043^T strain; 2, *R. lusitaniae* CBS 7604^T; 3, *Rh. colostri* CBS348^T. Symbols: +, positive; -, negative; w, weak; s, positive but slow; v, variable; n, no data.

Characteristics	1	2†	3†
Carbon compounds:			
L-Sorbose	+	+	-
Sucrose	-	-	+
Maltose	-	-	+
Cellobiose	-	v	+
Melezitose	-	-	v
Soluble starch	w	-	-
D-Xylose	w	+	v
L-Arabinose	s	-	-
D-Glucosamine	-	v	-
Ethanol	w	+	+
Glycerol	s	+	+
Galactitol	+	+	-
D-Mannitol	w	+	+
Metil-α-D-glucoside	-	-	v
Salicin	-	+	+
Succinate	-	+	+
Citrate	-	+	+
Saccharate	+	v	-
Xylitol	w	+	+
L-Arabinitol	w	n	n
Nitrogen compounds:			
Nitrate	+	+	+
Nitrite	s	+	+
Ethylamine	-	+	n
Other tests:			
Vitamin free	+	+	-
Urease	+	+	+
DBB reaction	+	+	+

†Data were taken from Kurtzman *et al*. (2011a).

Figure legends

Fig. 1. Phylogenetic analysis based on D1/D2 domain of the LSU rRNA gene from LLU043A^T and related species taken from GenBank (accession numbers in parentheses). Tree building was performed using the neighbor-joining method using the MEGA version 5.0 software package and evolutionary distances data were calculated according correction Kimura's two-parameter (Kimura, 1980). Values of less than 50% are not shown. Bar, 0.02 substitutions per nucleotide position.

Fig. 2. Cells morphology of *Rhodosporidium santurcensis* sp. nov. (LLU043A^T) after 6 days at 20 °C on 5% malt extract agar.

Fig. 1

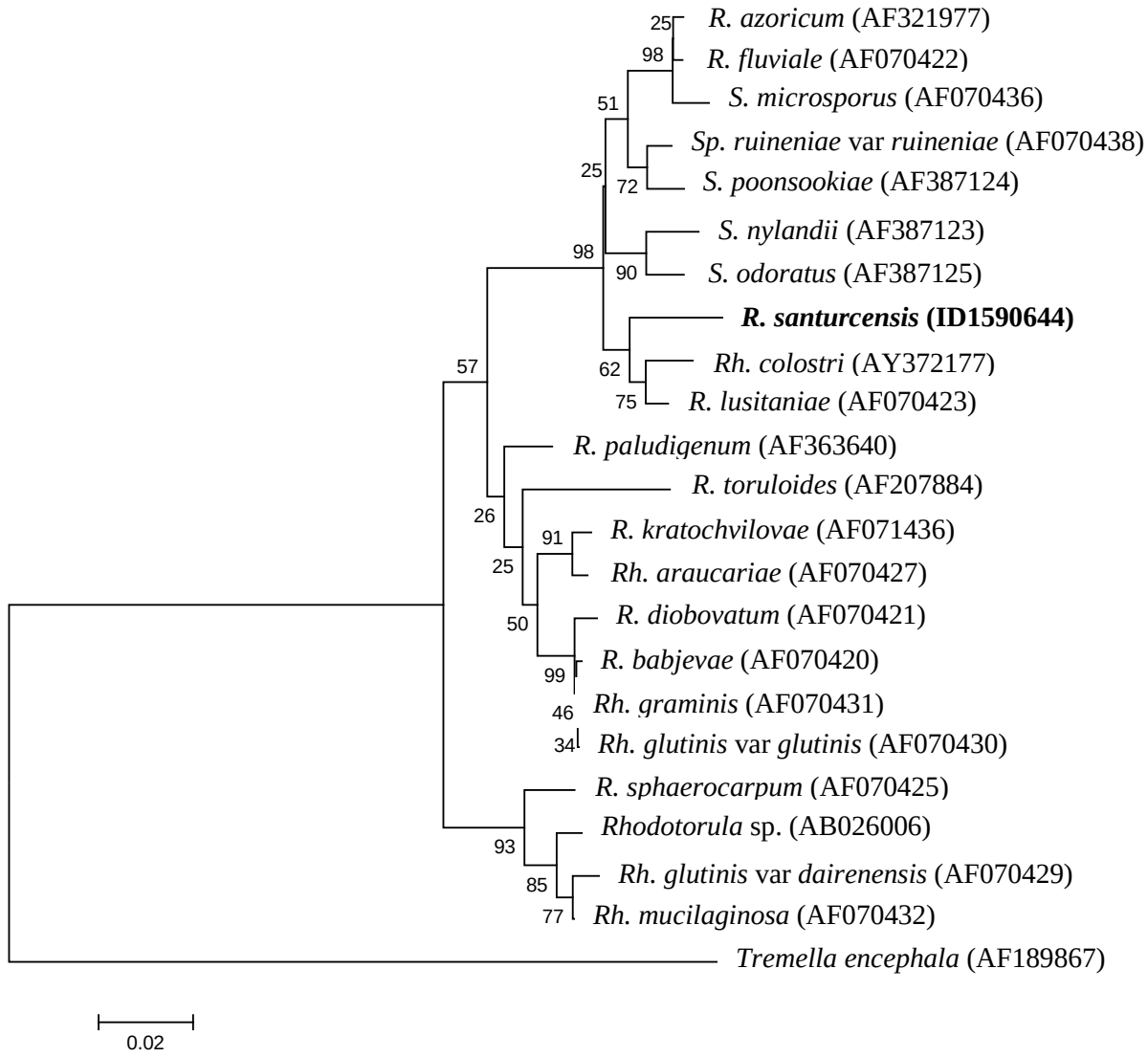
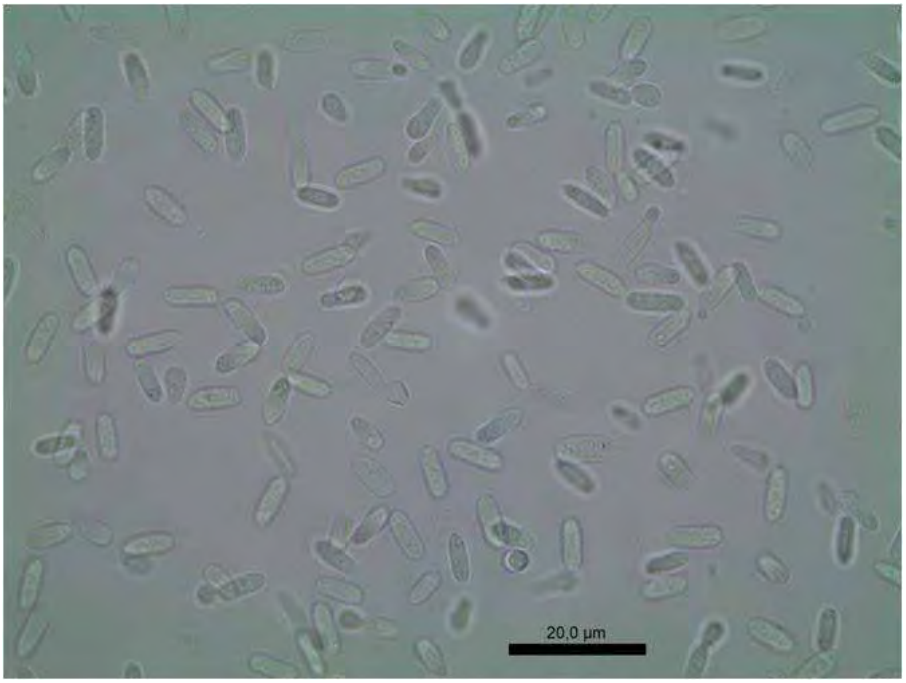

25
98
51
25
72
98
90
57
62
75
26
91
25
50
99
46
34
93
85
77
R. azoricum (AF321977)
R. fluviale (AF070422)
S. microsporus (AF070436)
Sp. ruineniae var ruineniae (AF070438)
S. poonsookiae (AF387124)
S. nylandii (AF387123)
S. odoratus (AF387125)
R. santurcensis (ID1590644)
Rh. colostri (AY372177)
R. lusitaniae (AF070423)
R. paludigenum (AF363640)
R. toruloides (AF207884)
R. kratochvilovae (AF071436)
Rh. araucariae (AF070427)
R. diobovatum (AF070421)
R. babjevae (AF070420)
Rh. graminis (AF070431)
Rh. glutinis var glutinis (AF070430)
R. sphaerocarpum (AF070425)
Rhodotorula sp. (AB026006)
Rh. glutinis var dairenensis (AF070429)
Rh. mucilaginosa (AF070432)
Tremella encephala (AF189867)
0.02

Fig. 2



5 SEGUNDA PARTE

FORMIGAS ATTINI DO BRASIL

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5.1 CAPÍTULO I

The fungus of the thelytokous fungus-growing ant *Mycocepurus smithii* (Formicidae, Myrmicinae) produces gongylidia-like structure

Target journal: PLoS ONE

Title: The fungus of the thelytokous fungus-growing ant *Mycocepurus smithii* (Formicidae, Myrmicinae) produces gongylidia-like structure

Running title: Gongylidia-like structures in the fungus garden of *Mycocepurus smithii*

Abstract

The fungus-gardening ants of the tribe Attini comprise a monophyletic group that has an obligatory mutualistic relationship with a basidiomycetous fungus which is believed to have evolved ca. 60-50 million years ago. The mutualism consists in the ants cultivating the fungus as their most important food source and in turn the ants provide nutrition, manure, and protection against the proliferation of alien microorganisms. The ant agricultures are divided into five groups: one of them is the group so-called the “Higher-attine” agriculture which cultivates the fungus *Leucoagaricus gongylophorus* considered as monoculture. Other group called “Lower-attine” agriculture cultivates a phylogenetically diverse set of lecocoprinaceous fungi. The Higher-attine fungi were always characterized by producing characteristic structures called gongylidia (swelling hyphae tips). These structures develop clusters forming the staphylae. This typical characteristic was considered as an evolutive trait and permitted to separate it from the other groups of attine ants. In this work, we present evidence that a fungus of Lower-attine agriculture can produce gongylidia-like structures and discuss the low partner fidelity between the culivated fungus and the ants.

Introduction

Mutualism, the symbiotic interaction between organisms in which each partner benefits, are widespread across the tree of life (Sachs et al. 2011). Many eukaryotes evolved obligate relationships with symbiotic organelles, such as mitochondria and/or chloroplasts, and provide stunning examples of ancient, evolutionary stable mutualisms (Blackstone 1994, Maynard Smith and Szathmary 1998, Timmis et al. 2004). Co-evolutionary processes, reciprocal genetic changes in one population in response to changes in the other species, shape these tight relationships, selecting for ecological specialization and resulting in co-diversification (Erhlich and Raven 1964, Benson et al. 1975, Janzen 1980, Herre et al. 1996, Pellmyr et al. 1996). Evolutionary traces of co-speciation can secondarily be inferred from matching patterns of phylogenetic histories (i.e., co-cladogenesis), but unfortunately, examples of currently occurring co-evolution, which could inform the selective processes and proximate mechanisms involved in establishing obligate interdependency between organisms, are inherently difficult to study, because the outcome of natural selection

remains often unknown at the time of study. However, the complex symbiosis of fungus-growing ants with leucocoprineaceous fungi and other associated microorganisms encompasses a diverse array of evolutionary transitions from ecological specialization to strict co-evolution, providing a well-suited system for studying the evolutionary origins of fungiculture in insects (Hinkle et al. 1994; Chapela et al. 1994; Mueller et al. 1998; Schultz and Brady 2008; Mehdiabadi et al. 2012).

The fungus-gardening ants of the tribe Attini comprise a monophyletic group of more than 290 described species (Brandão et al. 2011) which are distributed throughout the New World from Argentina in the south to the United States in the north (Kempf 1972, Weber 1972, Brandão 1991). All fungus-growing ant species rely obligately on basidiomycete fungi that are cultivated in underground chambers for food (Möller 1893, Wheeler 1907, Weber 1972, Quinlan and Cherret 1979). To facilitate the growth of the fungal symbionts, the ants provide nutrition, manure the fungus garden, and avoid the growth of alien microorganisms (Weber 1966, Weber 1972, Quinlan and Cherrett 1977, Pagnocca et al. 2012).

Based on their fungus-growing practices, the attine ants can be divided into five agricultural groups (Schultz and Brady 2008; Mehdiabadi and Schultz 2009). Group I: The so-called 'Lower-attine' agriculture includes the genera *Apterostigma, Mycocepurus, Myrmicocrypta, Cyphomyrmex* and *Mycetagroicus* that cultivate a phylogenetically diverse set of Lecocoprinaceous fungi from two distantly related fungal groups, the so-called clade 1 and clade 2 (Mueller et al. 1998). Lower-attine ants primarily collect dry leaf-litter, caterpillar feces, and occasionally seeds as substrate for their fungus garden (Leal and Oliveira 2000, Rabeling 2004, De Fine Licht and Boomsma 2010). Group II: In 'Higher-attine' agriculture *Trachymyrmex* and *Sericomyrmex* ants cultivate a clade of specialized fungi within clade 1 on a substrate consisting of dry leaf-litter, fallen leaves and fruits (Weber 1967, 1972, Feldmann et al. 2000). Group III: the genera *Atta* and *Acromyrmex* comprise the leaf-cutter ant agricultural system and cultivate a few closely related clones of fungi that may represent a single species of *Leucoagaricus gongylophorus* on a substrate of primarily freshly cut leaves and fruits (Silva-Pinhati et al. 2004; Scott et al. 2009; Mueller et al. 2010). GroupIV: coral fungus agriculture within the lower-attine ants, species in the *Apterostigma pilosum* species group that has switched to cultivate Coral fungi in the family Pterulaceae, which are unrelated to the leucocoprinaceous fungi cultivated by all other

fungus-growing ants (Villesen et al. 2004, Munkaski et al. 2004). Group V: yeast agriculture. Ant species in the *rimosus* species group of the genus *Cyphomyrmex* cultivate a clade 1 fungus in the single-celled yeast state instead of the mycelium state (Murakami and Higashi 1997, Mehdiabadi et al. 2012).

The attine ant-fungus mutualism evolved ca. 60-50 million years ago (mya) (Schultz and Brady 2008). Among the higher-attine ant cultivars no fungal isolates has been found free-living a part of without ants, which suggest the operation of a strict co-evolutionary dynamics (Mehdiabadi and Schultz 2009). Möller (1893) first described hyphal swellings in the fungus gardens of *Acromyrmex disciger*, which he called "Kohlrabikopf" due to its morphological similarity to cabbage turnip. Later Wheeler (1907) suggested the name "gongylidium, -a" (Greek= gongilis= turnip) for the same structure and Weber (1957) named the clusters of gongylidia, which Möller (1893) called "Kohlrabihaeufchen", "staphyla, -ae" (Greek= cluster of grapes). The presence of gongylidia is considered a unique characteristic of the higher attine and leaf-cutter ant symbioses (Weber 1966; Hölldobler and Wilson 1990; Chapela et al. 1994; Mueller et al. 2001, Schultz and Brady 2008, Solomon et al. 2011) being that there is no formal record of the presence of gonglylidia in fungus garden of lower attine ants. The gongylidia and staphylae are the food source for the queen and the larvae whereas the worker ants feed on plant sap, simple sugars produced from plant polysaccharide degradation, hyphae and staphylae as well (Möller 1893, Weber 1957, Littledyke and Cherrett 1976, Quinlan and Cherrett 1978, 1979, Angeli-Papa and Eymé 1985, Bass and Cherret 1995, Murakami and Higashi 1997, Silva et al. 2003). To determine the nutrient content of gongylidia, chemical analyses showed that they contain glucose, mannitol, trehalose, glycan, arabinitol, and glycogen, as well as lipids, and ergosterol (Martin et al. 1969; Quinlan and Cherret 1978, 1979; Mônaco Furletti and Serzedello, 1983), and free amino acids (Martin et al. 1969, Hölldobler and Wilson 1990). In contrast, the hyphae contain a high protein concentration but low concentration of lipids and carbohydrates (Martin et al. 1969; Quinlan and Cherret 1979).

The presence of gongylidia in the fungus garden of higher attine ants is considered an exclusively coevolved adaptation to the mutualism, because gongylidia are not known to convey any fitness benefits to the fungus unless it is cultivated by ants (Weber 1972, Mueller et al 1998, Mueller et al. 2001, Schultz and Brady, 2008; Solomon et al., 2011). Here we report the cultivation and maintenance of a gongylidia bearing fungus by the basal

fungus-growing ant *Mycocepurus smithii*. To test whether *M. smithii* cultivated a leafcutter fungus or lower attine cultivar, we genotyped the gongylidia bearing cultivar. Interestingly, the cultivar is a typical lower attine cultivar belonging to clade 1 of the tribe Leucocoprineae. *Mycocepurus smithii* reproduces thelytokously (i.e., via female parthenogenesis) in southeastern Brazil (Rabeling et al. 2009, 2011), and has previously been reported to cultivate a genetically diverse array of fungi (Mueller et al. 1998, Rabeling 2004, Vo et al. 2009), whereas other basal attine species are known to be faithful to a single cultivar lineage (Schultz et al. 2002, Mehdiabadi et al. 2012). We discuss whether asexual reproduction of *M. smithii* may have prompted the cultivation of morphologically and genetically diverse cultivars.

Material and Methods

To study the population biology of *M. smithii*, we excavated nests of *M. smithii* on the Campus of São Paulo State University in Rio Claro (22°23'48.81"S; 47°32'39.89"W), São Paulo, Brazil on the 15th of July 2011. The nest had a single nest entrance and the excavation followed the methodology described in Rabeling et al. (2007, 2009). The nest consisted of two chambers at 25 cm and 53 cm depth, respectively. The first chamber was empty and the second chamber (collection code: CR110715-02) contained the fungus garden hanging from the ceiling. The second chamber had a diameter of 3cm and the mycelium filaments of the fungus garden were 2cm long (Fig. 1a). The ant colony and the fungus garden were collected alive and placed into a laboratory nest for further observation. The lab nest consisted of a plastic container with plaster of Paris bottom (see Schultz 1993 for lab nest setup). Ants were identified using a Leica MS5 stereomicroscope and voucher specimens were deposited in Maurício Bacci's Molecular Evolution Laboratory at São Paulo State University in Rio Claro, and Christian Rabeling's collection.

To compare the gongylidia-like structures of the *M. smithii* garden to gongylidia of leafcutter ant fungi, we collected colonies of three leafcutter ant species (*Atta laevigata*, *Atta sexdens*, and *Acromyrmex disciger*) and one *Trachymyrmex* species, *T. fuscus*, on Campus of São Paulo State University on September 13, 2011.

Macro and microscopic observations

The presence of gongylidia in *M. smithii* gardens was first noted during observations of the live colony with a Leica MZ16 stereomicroscope and confirmed under higher magnification with a Leica ICC50 brightfield microscope. The *M. smithii* colony (CR110715-02) was maintained in a dark room at 25°C during two days. Samples of the fungus garden were taken and observed under a Leica MZ16 stereomicroscope. Measures of gongylidia where taken with the Leica Application Suite V3 program in a brightfield microscope Leica ICC50.

To compare whether there was difference in the gongylidia size among *M. smithii* and higher attine cultivars, samples of staphylae were taken to measure the diameter of gongylidia (n=40 per colony per ant species). For microscopic studies, the staphylae were removed with a pair of acupuncture needles and placed on a slide with a drop of glycerin 15%. To test for variance of size distribution in gongylidia collected from *M. smithii*, *Trachymyrmex* and leafcutter ant cultivars, we conducted a variance analysis (one-way ANOVA and Tukey test) with probability level (P) less than 1% ($P<0.01$) using the software package BioEstat 5.0 (Ayres et al. 2007).

Genotyping and molecular phylogenetic analyses

To extract genomic DNA of the gongylidia bearing *M. smithii* garden, we took tissue samples of staphylae following the methodology described by Martins Jr et al. (2007). The ITS region was amplified according to Manter and Vivanco (2007) using the primer pairs: forward ITS 5 [GGAAGTAAAAGTCGTAACAAGG] and reverse ITS 4 [TCCTCCGCTTATTGATATGC] (White et al 1990). The reaction program consisted of an initial 2min incubation at 96°C, followed by 28 cycles of 46s at 96°C, 30s at 50°C and 4 min at 60°C. The PCR product was gel-purified using an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). For the sequencing reaction, we used the same primers as for the amplification. The sequencing reaction was prepared with 100 ng of PCR template, 6 pmols primers, 2.0 μl BigDye Terminator (Applied Biosystems), 1,0 μl buffer (200 mM Tris.HCl, 5 mM $MgCl_2$) and ddH_2O. Sequencing products were purified and then analyzed on an automated sequencer ABI3500 (Applied Biosystems). The consensus sequence was edited with the program BIOEDIT 7.0.5 (Hall, 1999) and aligned using

CLUSTALW (Thompson et al., 1994). The obtained sequence was compared to sequences deposited in the NCBI databases (www.ncbi.nih.gov) and was deposited at NCBI GenBank under the accession number JK027477.

Results

As a result of observations on fungus garden of *M. smithii* (CR110715-02) with naked eye, stereomicroscope (Fig. 1b) and microscope (Fig. 1c, d) it was possible to confirm and document the presence of staphylae-like structures. These structures consisted by clusters of dilated hyphal tips with the same appearance of true gongylidia which are typical structures formed by the cultivated fungus of higher attine ants. Because there are no previous studies of these structures in cultivated fungus of this lower attine ant and due to similar appearance of gongylidia from fungus of higher attine, we decided to call them as "gongylidia-like structures" hereafter.

The general morphology of gongylidia-like structures from *M. smithii* fungus is similar to true gongylidia of higher attine ants with a few difference in the size and cytoplasmatic appearance. Morphological analysis (Table 1) showed that gongylidia-like were the smallest within the group (Fig. 1e, f, g, h). No vacuoles or cytoplasmatic condensations were observed in gongylidia-like structures from *M. smithii*, in contrast with the observed inside of the gongylidia from the fungus of *A. sexdens* (Fig. 1h) and *A. laevigata* (Fig.1g), respectively. In this feature, gongylidia-like structures from *M. smithii* fungus is similar with gongylidia of *T. fuscus* (Fig. 1e) and *Acr. disciger* (Fig. 1f).

Insert Table 1

To test whether the gongylidia bearing *M. smithii* fungus gardens are closely related to leafcutter ant cultivars, or whether gongylidia arose independently in basal attine cultivars, we conducted molecular phylogenetic analyses of the ITS region of the *M. smithii* cultivar to determine its phylogenetic position. Both Bayesian and Maximum Likelihood analyses (data not shown) revealed that the gongylidia bearing *M. smithii* garden of the Rio Claro population is embedded in the co-called clade 1 of leucocoprineous fungi, which is a typical representative of basal attine cultivars, which indicates that gongylidia or gongylidia-like structures evolved independently in leafcutter ant cultivars and basal attine cultivars.

Discussion

The morphological analysis between gongylidia-like structure from fungus garden of *M. smithii* and true gongylidia from four higher attine showed a different size but similar external appearance including the formation in clusters as staphylae and not dispersed throughout the fungus garden. Taking into account these characteristics is possible to think that they are homologous structures.

Nevertheless, in the previous observations of staphylae in higher attine nests, Spegazzini (1922) suggested that the formation is stimulated by the constant pruning practiced by the ants. Similar conclusion was pointed by Bass and Cherret (1996) who correlated this type of behavior with the increased production of these structures. On the other hand, Powell and Strandling (1986) showed that among the factors that could affect the development of gongylidia are the nature of the substrate, pH and temperature leading to differences in number and in the size of them.

In most of the attine ants the mutualistic fungus is vertically transmitted to next generation as a consequence of the nuptial flight (Huber 1908, Weber 1972b). There are two kinds of populations within group of *M. smithii* species, with sexual reproduction and with parthenogenetic reproduction (Rabeling et al 2011). In the case, there is no sexual reproduction, and thus their mutualistic fungi is not propagated in nature by this way (Fernández-Marín et al 2005, Rabeling et al 2009, Rabeling et al 2011).This suggests *M. smithii* ants may have “plasticity” in the selection of cultivated fungi discriminating different fungi which result genetically different. Overall, this species may have domesticated several fungal partners, a trait confirmed by the genetic variability found between the different strains (Mueller et al 1998, Vo et al 2009; Rabeling 2004). Thus, it is possible to suppose that *M. smithii* shows to have a partner choice cooperation with their cultivated fungi differing from the other fungus growing ants which have high partner fidelity (Schultz et al. 2002; Scott et al. 2009; Mehdiabadi et al. 2011).

Unfortunately there are not enough data for comparing sequences cultivars of the sexual and asexual populations of *M. smithii*, a trait that could shed light in this amazing symbiosis.

Acknowledgements

The authors are gratefully to CNPq and Fapesp for financial support. VEM is recipient of a CAPES/PEC-PG scholarship.

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Table 1. Comparison among sizes of "gongylidia-like structures" from fungus of *M. smithii* and true gongylidia from fungus of four higher attine ants

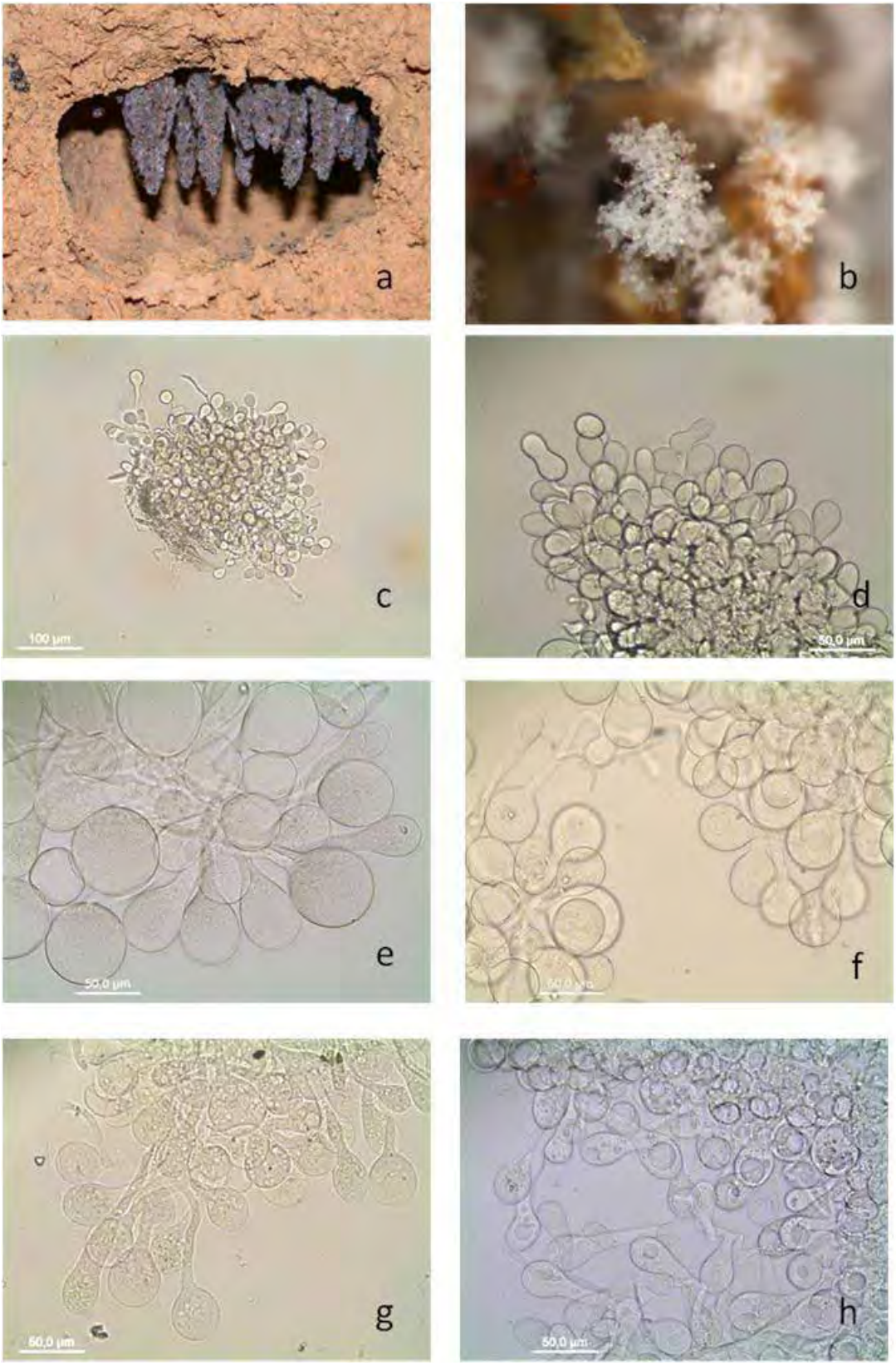
ant species	range diameter (μm)	mean ± SD
M. smithii	16.30 - 25.41	$20.25^{a} \pm 2.50$
T. fuscus	42.01 - 68.26	$54.60^{b} \pm 5.93$
Acr. disciger	32.32 - 52.63	$41.50^{c} \pm 4.47$
A. laevigata	30.21 - 57.20	$39.67^{c} \pm 6.17$
A. sexdens	21.04 - 39.99	$31.72^{d} \pm 4.02$

NOTE: Means with different letters are significantly different (Tukey, $P<0.01$)

Legends

Fig. 1. (a) Typical chamber and fungus garden of the nest of *M. smithii* (b) Staphylae showing gongylidia in the fungus garden of *Mycocepurus smithii* (CR110715-02) (x8) (c) Staphylae of *M. smithii* (CR110715-02) (x200) (d) Gongylidia of *M. smithii* (CR110715-02)(x400) (e) Gongylidia of *T. fuscus* (x400) (f) Gongylidia of *Acr. disciger* (x400) (g) Gongylidia of *A. laevigata*(x400) (h) Gongylidia of *A. sexdens* (x400)

Fig. 1



5.2 CAPÍTULO II

Escovopsis brasiliensis sp. nov., isolated from a nest of *Mycocepurus goeldii* Forel

Target journal: Mycologia

Short title: New species of *Escovopsis*

Title: *Escovopsis brasiliensis* sp. nov., isolated from a nest of *Mycocepurus goeldii* Forel.

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Abstract:

Currently, there are two formal descriptions of species in the genus *Escovopsis* and the existing literature defines it as a specialized mycoparasite from the nests of Attini ants (Hymenoptera: Formicidae). These two species are: *E. weberi* Muchovej and Della Lucia isolated from nests of *Atta* sp. in Brazil and *E. aspergilloides* Seifert, Samson and Capela from *Trachymyrmex ruthae* from Trinidad. We described in this work a new species, *Escovopsis brasiliensis* isolated from a nest of *Mycocepurus goeldii*, which differs from the two other species by the conidiophores, conidiogenous cells and conidia morphology. Another characterist is that this new species produce enteroblastic conidia.

Key words: Hypocreales, fungus-growing ant, *Escovopsis weberi*, *Escovopsis aspergilloides*, Ascomycetes

INTRODUCTION

Ants in tribe Attini (Hymenoptera: Formicidae) are known as “fungus-growing ants” because they cultivate basideomycetous fungi (Agaricales) for food (Weber 1972, Littledyke and Cherrett 1976, Quinlan and Cherrett 1979, Bass and Cherrett 1995). According to the type of fungus cultivated by attine ants Schultz and Brady (2008) divided the attine fungiculture in 5 groups: (i) the lower agriculture of the genera *Mycocepurus*, *Myrmecocripta* and some *Apterostigma* species) in which the fungus belongs to tribe Leucocoprineae (group G3) (ii) the coral fungus agriculture (Pterulaceae) (groups G2-G4) (*Apterostigma*, “pilosum group”); (iii) the yeast agriculture of the lower attines in the *Cyphomyrmex*, “rimosus group”) in which the leucocoprineaceous fungi belong to the (G3); (iv) the generalized higher agriculture of the non-leaf-cutting ants (*Trachymyrmex* and *Sericomyrmex*) (G1) and (v) the leaf-cutter agriculture (*Atta* and *Acromyrmex*) in which both genera cultivate different fungi belonging to the tribe Leucocoprineae (G1).

It is believed that this ancient symbiosis originated at 60 to 50 million years (Schultz and Brady 2008). A plethora of micro-organisms is continuously introduced into the fungus gardens and even being the symbiosis an open system the ants are usually well succeded in the maintenance of the cultivar (Pagnocca et al. 2012). However, especially in unbalanced nests, a hypocrealean fungus in the genus *Escovopsis* is considered a specialized mycoparasite that may threat the symbiosis (Currie et al. 1999, Reynolds and Currie 2004). Apparently, the prevalence of this fungus is higher in nests of Attini ants from Central America (Currie et al. 1999; Gerardo et al. 2006) compared with those of South America (Rodrigues et al. 2005, 2008).

Escovopsis was observed by the first time by Möller (1893) who found two particular fungi growing associated with the fungal cultivars of *Acromyrmex disciger* and *Apterostigma* ant species in Blumenau, SC, Brazil. Kreisel (1972) also found one of these fungi in nests of *Atta insularis* in Cuba and formally described it as *Phialocladus zsoltii*. However, Muchovej and Della Lucia (1990) renamed the genus as *Escovopsis* and the species as *E. weberii* (in honor of Neil Weber, the famous American entomologist). The other fungus observed by Möller (1893) in nests of *Apterostigma* species (*Ap. wasmannii*, *Ap. pilosa* and *Ap. moelleri*) had similar morphology to *Aspergillus* but it was not formally described and named. However, Seifert et al. (1995) rediscovered this fungus in nests of *Trachymyrmex ruthae* from Trinidad and described it as *Escovopsis aspergilloides* by its

morphological similarity with *Aspergillus*. Since 1995, there is no report of species description belonging to the genus *Escovopsis*.

This paper addresses the description of a new species of *Escovopsis* based on a polyphasic study. The name *Escovopsis brasiliensis* is proposed to designate the new species isolated from a nest of *Mycocepurus goeldii* Forel, a basal fungus-growing ant.

MATERIALS AND METHODS

Sampling site.– The fieldwork was carried out on August, 13th, 2011 at the campus of the São Paulo State University – UNESP (22°23'46.93"S, 47°32'40.12"W), Rio Claro, São Paulo, Brazil. The samples were collected from the fungus garden of a *Mycocepurus goeldii* Forel nest.

Fungal isolation.– Small fragments of the fungus garden of *M. goeldii* were removed with sterile forceps and placed in 9 cm diam Petri dishes containing PDA (potato dextrose agar) with chloranphenicol (Sigma) 200 $\mu g.mL^{-1}$ and incubated at 25 C/ 10 days in the dark.

Mycelial growth.– Radial growth, conidia and chlamydospores formation and pigment production were determined on three different culture media without antibiotics: oatmeal agar (OA), 2% malt extract agar (MEA), and potato dextrose agar (PDA) at seven different temperatures (between 5 and 35 C with 5 C intervals). Assays were performed in triplicate and lasted 2 wk. For the micro-morphology the strain was grown on 2% MEA after 7 d at 25 C.

DNA extraction, PCR and sequencing.– Fungal mycelia were grown on PDA at 25 C during 8 d. Genomic DNA extraction were performed as described in Almeida (2005) after breaking the mycelia with a mortar and pestle in liquid nitrogen. It was amplified a single exon of the *tef1-α* gene with the *Escovopsis*- specific pair primers EF6-20F (5'-AAGAACATGATCACTGGTACCT-3') and EF6-1000R (5'-CGCATGTCRCGGACGGC-3') according to previous studies on *Escovopsis* phylogeny (Currie et al. 2003, Gerardo et al. 2004, Taerum et al. 2007, Taerum et al.2010). PCR was performed with initial denaturation at 96 C for 3 min, followed by 35 cycles at 96 C for 1 min, 61 C for 1 min, and 72 C for 1 min. PCR products were purified with GFX DNA and Gel Band Purification Kit (GE Healthcare). Sequencing reaction was performed with ABI Prism® Big Dye®Terminator

v3.1 Cycle Sequencing Kits using the same primers as in PCR amplification and placed in a 3130 Genetic Analyzer (Applied Biosystems). Sequence was assembled in BioEdit v. 7.0.5.3 (Hall 1999).

Phylogenetic analysis.– For the phylogenetic analysis representative *tef1-α* gene sequences of three families of Hypocreales and *Escovopsis* species were selected and downloaded from GenBank. DNA sequences were aligned with MAFFT version 6 (Katoh and Toh 2008). The aligned dataset was analyzed with maximum likelihood (ML) MEGA 5.05 phylogenetic software package (Tamura et al. 2011). The distances between the sequences were based on Kimura 2-parameter model (Kimura 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (1000 replicates) (Felsenstein 1985).

RESULTS

During the isolation procedures two different fungal strains were isolated in PDA. One was identified as the mutualist fungus of *M. goeldii* (GenBank acession: xxxxxx) with similarity of 99% with the mutualistic fungus of *Mycetagroicus cerradensis* (GenBank HM245775). This finding is in agreement with the survey of Solomon et al. (2011) who reported that they are nested in the group of lower attine cultivars (G3-clade2). The other strain isolated from fungus garden of *M. goeldii* showed white colonies that later turned yellow and after brown and we considered to be an undescribed strain in the genus *Escovopsis*. We propose the name *Escovopsis brasiliensis* for this strain.

TAXONOMY

Escovopsis brasiliensis Cabello, Masiulionis, Rodrigues & Pagnocca FIG. 1

MycoBank accession: xxxxx

GenBank accession: XXXX

Etymology: brasiliensis, referring to Brazil, country where the species was first found.

Coloniae in agaro maltos (2%) post 7 dies 9cm diam, margine difusa irregulari. Mycelium aerium sparsum, albidum, reversum pallidum. Conidiophora simplicia vel repetite ramosa, usque ad 230µm alta, 4.5–5µm crassa, rami terminalis superne fértiles Cellulae conidiogenae anguste ampuliformes, hyaline 10–20µm x 7–8.5µm, Conidia anguste ovata, basi truncata, apice rotundata, 3.75–4.5x 2.30–3 µm, manifeste verrucosa, verrucae ≤ 1 µm (650nm–112 µm) modice ochracea, non-catenata.

Holotypus herb. CBMAI- 186/2012 - Isolates ex nido *Mycocepurus goeldii* in Rio Claro, São Paulo, Brazil (22°23'46.93"S, 47°32'40.12"W) (vivus sigla de CBMAI- 186/2012 et LPS Cul Noxx)

Colonies on 2% MEA after 7 d at 25 C filling a 9–cm Petri dish, margin diffuse, uneven. Aerial mycelium sparce, reverse pale, conidiophores simple or repeat branched, up to 230µm long, 4.5–5µm thick, terminal branches fertile, conidiogenous cells ampuliform, colorless 10-20µm x 7–8.5µm.

Conidia ovate with truncate base, apex rounded, 3.75–4.5x 2.30–3µm, clearly verrucose, warts ≤ 1µm (1–650nm, 12 crowded) slightly ochraceous, single (not in chains).

Cultures. – *Escovopsis brasiliensis* was isolated from the upper parts of fungus garden of *Mycocepurus goeldii*. *E. brasiliensis* grows on all agar media and at six temperatures tested but no growth at 35 C. The optimum range of growth was on the interval of 25 and 30 C. The growth at 5 and 10 was observed but after 2 and 1wk, respectively.

The growth (mm) after 72 h of incubation at the temperature range of 15–30 C in OA, MEA and PDA are shown in TABLE 1. The fungus grows well in a temperature range spanning from 15–30 C. The highest values were obtained in the range of 25-30 C. Conidia were observed after 3 d in all the temperatures tested in PDA; after 4 and 12 d at 20–30 C in OA and MEA, respectively. Conidiation in PDA is white at beginning becoming yellow in the center and turn to brownish-yellow after 5 d at 25 and 30 C (FIG. 2, 3). Chlamydospores were observed after 4 days in the temperature range of 15–25 C in MEA and PDA and at 30 C in OA medium. Aerial mycelia and the occurrence of stolons-like hyphae were also observed regularly. The reverse plate is colorless on MEA but yellow on OA and PDA after 2–3 d at 25–30 C (FIG. 2, 3). On three media no exudate or odor were produced. The

secretion of yellow pigment was observed on OA and PDA after 2 d at 10, 15, 20, 25 and 30 C (FIG.2).

Distribution. – Rio Claro, São Paulo, Brazil (22°23'46.93"S, 47°32'40.12"W).

Habitat. – Isolated from fungus garden of *Mycocepurus goeldii* (Hymenoptera: Formicidae: tribe Attini).

Phylogenetic analysis.– As a result of the comparison of the DNA sequences of tef1-α domains of tef1-α nuclear gene from *Escovopsis brasiliensis* sp. nov. and those retrieved from GenBank we found that the closest relative is the strain DQ848209 isolated from the fungus garden of *Cyphomyrmex longiscapus* which showed 96% of identity (27 different residues, 16 transversions and 21 transitions). The other similar strain (DQ 848167) showed 95% of identity (39 different residues, 14 transversions and 25 transitions) and was found in the fungus garden of *A. dentigerum*.

The new species described in the present work belongs to the genus *Escovopsis* according to the phylogenetic analysis using the tf1-α gene marker (FIG. 4). The analysis indicated two major clades of *Escovopsis* each representing *Escovopsis* associated with higher and lower attine ants. *E. brasiliensis* is accommodated in a somewhat separate position (although with lower bootstrap support) in the clade that harbor *Escovopsis* from lower attine ants (FIG. 4).

DISCUSSION

Currently, only two *Escovopsis* species are recognized, *E. weberi* Muchovej and Della Lucia and *E. aspergilloides* Seifert, Samson and Chapela. However, considering the several and variable DNA sequences deposited in GenBank and Mycobank likely the genus has many undescribed strains. Our strain differs from the two other species in the genus, *E. weberi* and *E. aspergilloides*, by the conidiophores, conidiogenous cells and conidia morphology. The holoblastic ontogeny of the conidium is shared by the new species *E. brasiliensis* and *E. weberi* according to the analysis made by Muchovej and Della Lucia (1990) for the last species. On the other hand *E. aspergilloides* produce enteroblastic conidia. *E. brasiliensis* produce branched conidiophores (up to 230µm long and 4.5µm

wide). From the main axe side branches are developed, almost in right angles. In turn of every branches new fertile blanches arise. Branches are covered with discrete sporogenous cells (10–20µm x 7–8.5µm) with no trace of vesicules. *E. weberi* has terminal and sessile sporophores (43–58µm long and 11.5–14µm wide), covered with discrete conidiogenous cells (3–4.5 wide 4.5 µm long.). In *E. aspergilloides* the conidiophores are polycephalous on stipes up to 1350µm long and resulting in succession of vesicles; vesicles have a uniseriate layer of phialides, similarly to *Aspergillus*. Conidium of *E. weberi* is globose to ovoid, smooth and basipetalously catenate, 2.2–3.3 x 2–3µm. In *E. aspergilloides* conidia are ellipsoidal, 2.5–3.7 x 2µm and produced in chains. Finally in *E. brasiliensis* conidia are ovoid, the base distinctly truncate and coarsely verrucose, bigger that those from previous species ranging 3.75–4.5 x 2.30–3µm and the conidia are not produced in chains.

The genus *Escovopsis* have been found associated exclusively with nests of Attini ants and according to Currie et al. (2001) this fungus is prevalent in the bottom parts of fungus garden (exhausted substrate) and our strain of *E. brasiliensis* was isolated from young parts of the garden.

Because *E. brasiliensis* was isolated from a lower attine ant that cultivates G3 fungus, it was expected that the parasite from the same nest would cluster with similar *Escovopsis* that infects G3 fungi. In fact, we observed that *E. brasiliensis* clustered in a somewhat separate position but between *Escovopsis* isolated from gardens of *A. dentigerum* and *C. longiscapus*, two lower attine ants (FIG. 4). *Escovopsis* that infects lower attine cultivar appears to be very diverse in both genetic and morphological markers (Gerardo et al. 2006).

Little is known about microbial community from fungus garden of lower attine ants such as *M. goeldii*. More studies are necessary to elucidate the microbial ecology and the real role played by these microorganisms in the fungus garden of these ant nests.

ACKNOWLEDGMENTS

We acknowledge O.C. Bueno for permission to use the SEM of Department of Biology, UNESP and A. Teruyoshi Yabuki for the aid during the SEM photographs session. V. E. Masiulionis was sponsored by a scholarship from CAPES/PEC-PG. This work was funded

by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq – Brazil) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

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TABLE I. Growth diameter (mm) after 72 h of incubation at temperature range of 15-30 C on different culture media

Culture media	Temperature C			
	15	20	25	30
OA[1]	25-40	30-40	80-81	80-81
MEA[2]	30-35	45-50	65-72	65-72
PDA[3]	25-30	45-50	65-72	65-72

[1]OA: oatmeal agar; [2]MEA: malt extract agar; [3]PDA: potato-dextrose agar

LEGENDS

FIG. 1. *Escovopsis brasiliensis.* (a-d, f) Morphology of conidiophores, and conidia, (e,g) chlamydospores. Growth on PDA at 25 C after 7 d (Electron microscope TM300 Tabletop-Hitachi).

FIG. 2. Growth of *Escovopsis brasiliensis* on PDA at 25 C after 5 d (a), 7 d (b) and 9 d (c). Front and reverse of Petri dishes.

FIG. 3. *Escovopsis brasiliensis*. (a-c) Conidiophores (x400), (d-e) Conidia (x400), (e-f) Chlamidospores (x400; x1000). Optical microscope Leica ICC 50 (x400).

FIG. 4. Maximum likelihood tree inferred from an alignment of the *tef1-α* gene showing the phylogenetic position of *Escovopsis brasiliensis* and additional *Escovopsis* strains. The phylogenetic tree was inferred under the Kimura 2-parameter substitution model. The number on branches are bootstrap support (>50%) obtained in 1,000 bootstrap pseudoreplications. *Escovopsis* sequences are named after the ants species were they were isolated from. The Genbank accession number or code are in parentheses. Bar, 0.02 substitutions per nucleotide position.

FOOTNOTES

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FIG. 1

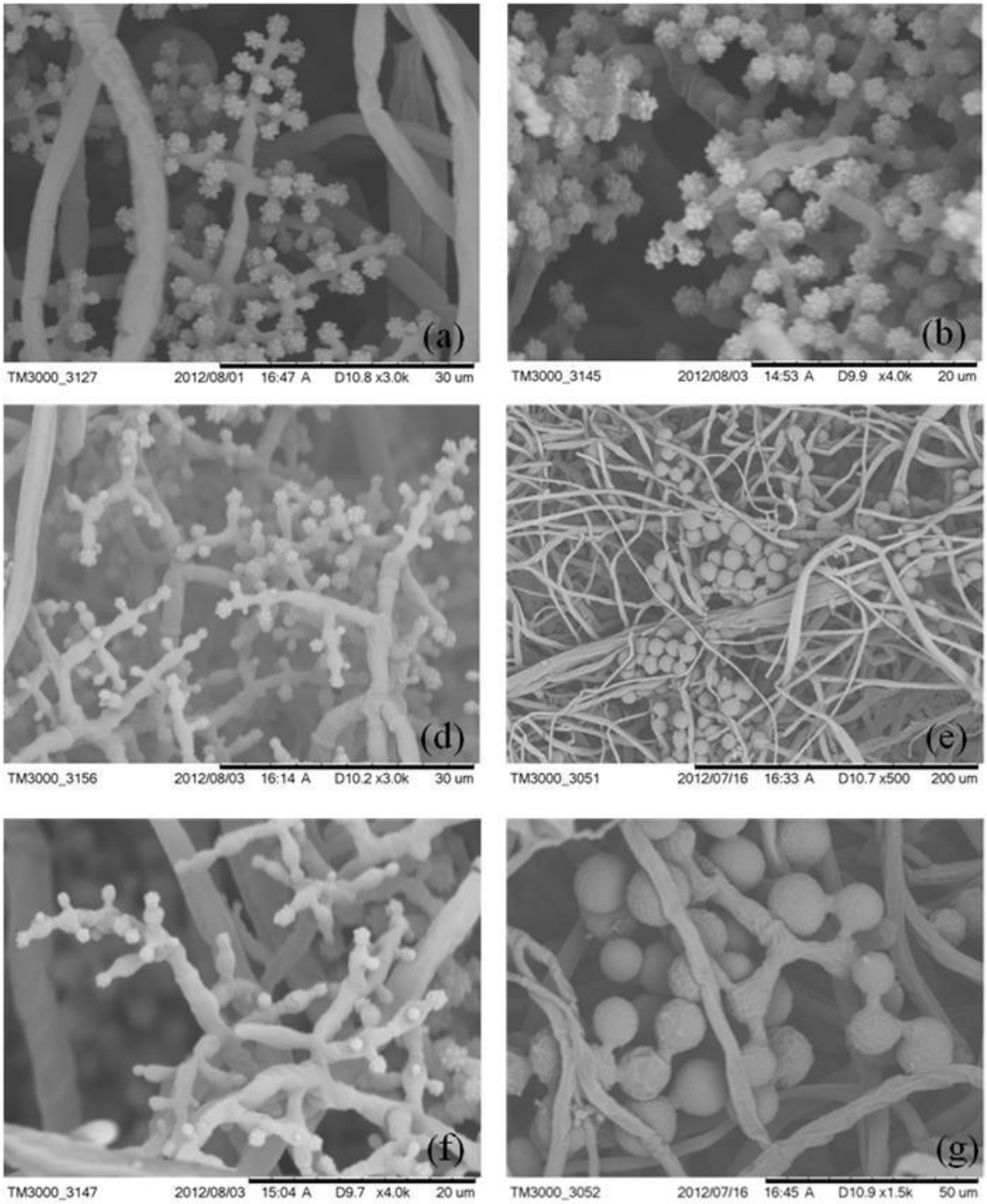

(a)
TM3000_3127 2012/08/01 16:47 A D10.8 x3.0k 30 um
(b)
TM3000_3145 2012/08/03 14:53 A D9.9 x4.0k 20 um
(d)
TM3000_3156 2012/08/03 16:14 A D10.2 x3.0k 30 um
(e)
TM3000_3051 2012/07/16 16:33 A D10.7 x500 200 um
(f)
TM3000_3147 2012/08/03 15:04 A D9.7 x4.0k 20 um
(g)
TM3000_3052 2012/07/16 16:45 A D10.9 x1.5k 50 um

FIG. 2

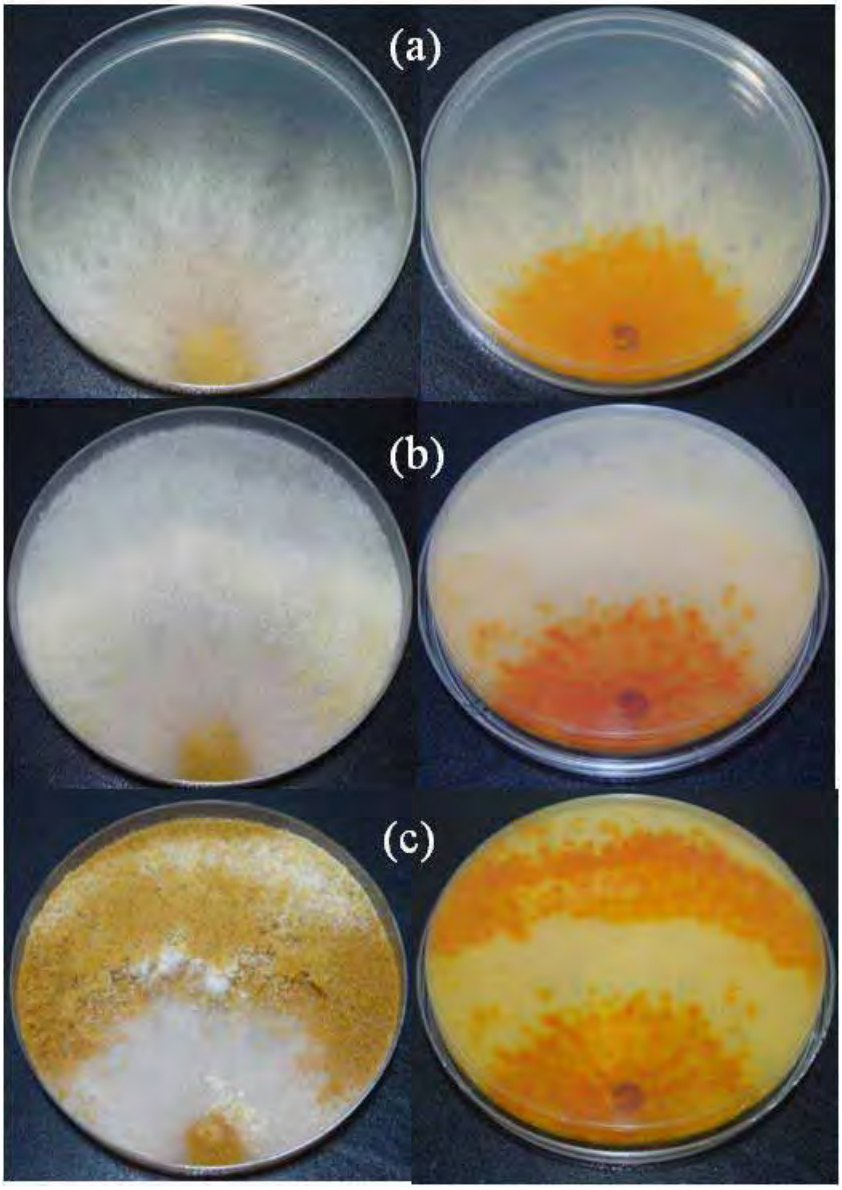


FIG. 3

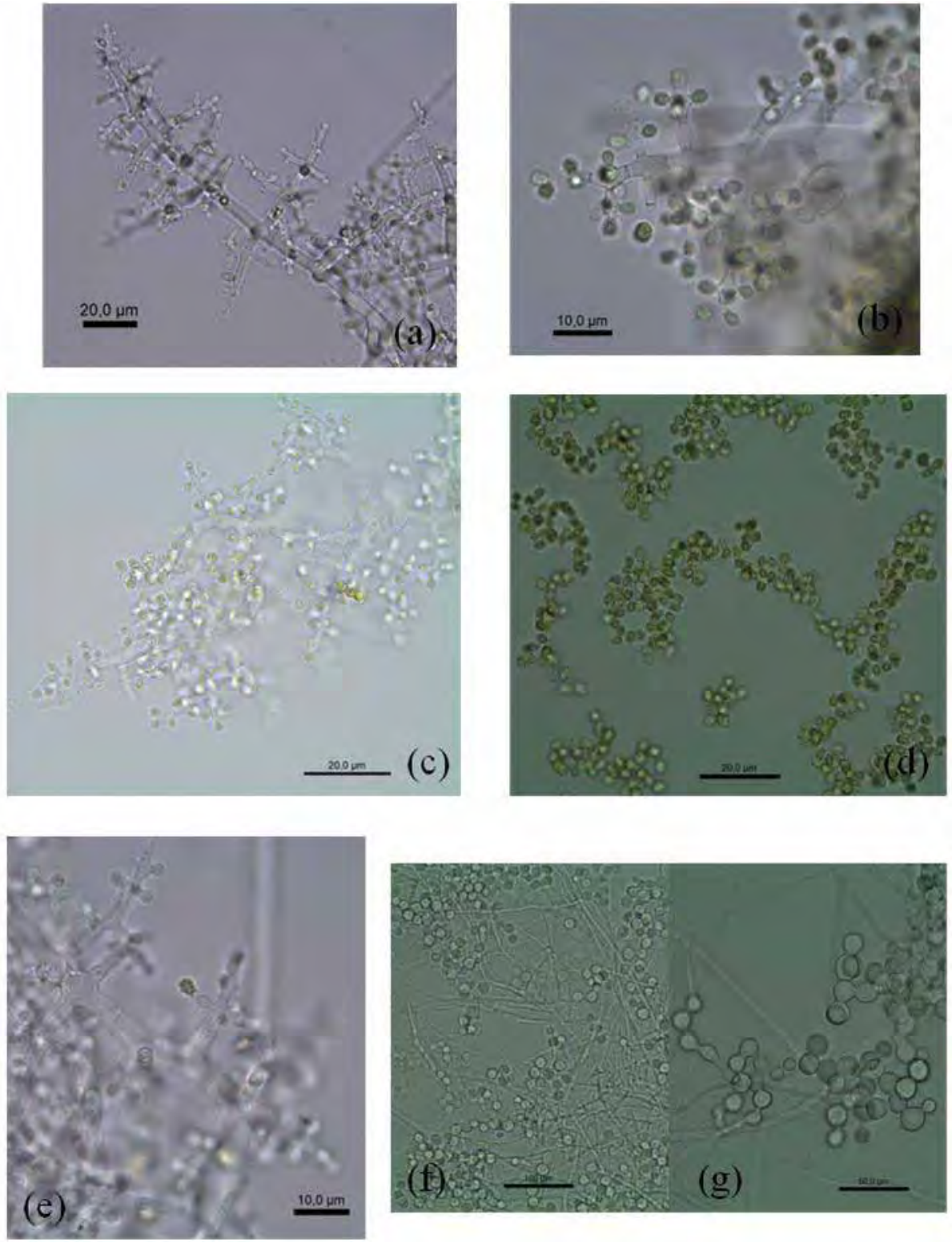
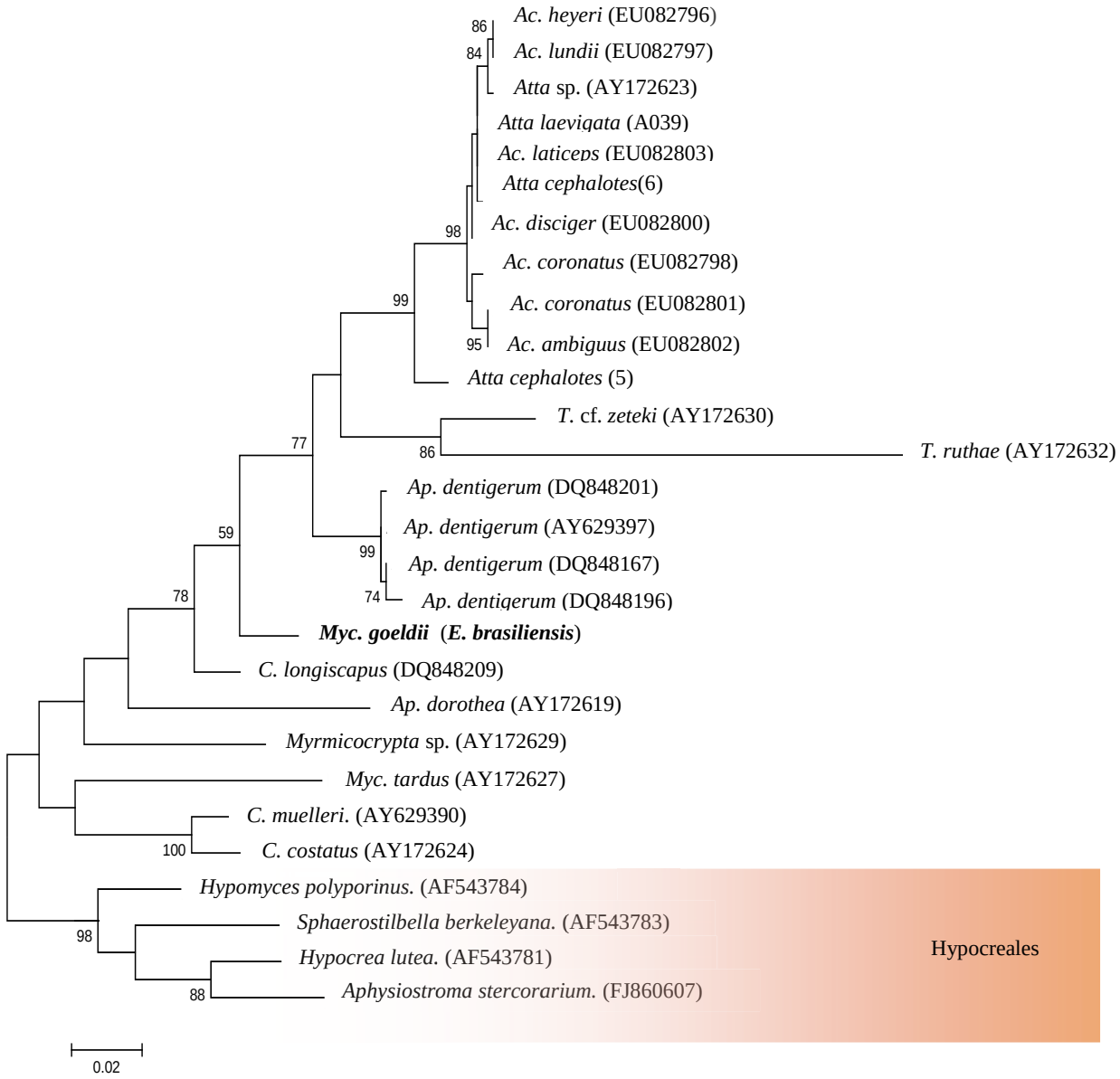


FIG. 4


Ac. heyeri (EU082796)
Ac. lundii (EU082797)
Atta sp. (AY172623)
Atta laevigata (A039)
Ac. laticeps (EU082803)
Atta cephalotes(6)
Ac. disciger (EU082800)
Ac. coronatus (EU082798)
Ac. coronatus (EU082801)
Ac. ambiguus (EU082802)
Atta cephalotes (5)
T. cf. zeteki (AY172630)
T. ruthae (AY172632)
Ap. dentigerum (DQ848201)
Ap. dentigerum (AY629397)
Ap. dentigerum (DQ848167)
Ap. dentigerum (DQ848196)
Myc. goeldii (E. brasiliensis)
C. longiscapus (DQ848209)
Ap. dorothea (AY172619)
Myrmicocrypta sp. (AY172629)
Myc. tardus (AY172627)
C. muelleri. (AY629390)
C. costatus (AY172624)
Hypomyces polyporinus. (AF543784)
Sphaerostilbella berkeleyana. (AF543783)
Hypocrea lutea. (AF543781)
Aphysiostroma stercorarium. (FJ860607)
Hypocreales
86
84
98
99
95
77
86
59
99
78
74
100
98
88
0.02

6 CONSIDERACIONES FINAIS

Embora a Argentina possua uma grande diversidade de formigas Attini, elas foram pouco estudadas até o momento, especialmente em relação à microbiota que sabidamente está presente em seus ninhos. Nesta pesquisa, desenvolvida nos últimos 4 anos, procuramos conhecer um pouco mais sobre esse grupo de insetos. Especificamente, estudamos três espécies de formigas cortadeiras de uma região da província de Santa Fé, concentrando nossos esforços no conhecimento da microbiota associada. Fizemos um estudo das comunidades de leveduras provenientes dos ninhos de *A. heyeri*, *A. lobicornis* e *A. lundii*, das quais, as duas primeiras são consideradas praga na província de Santa Fé e a terceira é uma espécie praga típica da província de Buenos Aires. Como decorrência, duas espécies novas de leveduras estão sendo propostas e deverão ser descritas em breve.

Durante um de nossos trabalhos de campo pudemos registrar, em *A. lobicornis*, o forrageamento de um fungo basidiomiceto do tipo coprófilo, *Psilocybe coprophila*, um fenômeno que à época da observação (janeiro de 2010) era desconhecido. Curiosamente, quase ao mesmo tempo, um registro semelhante também foi observado na Argentina, com outra espécie, *A. lundii*, a qual foi encontrada forrageando o fungo *Agrocybe cylindracea*, o qual crescia sobre o caule de uma planta do gênero *Populus*. Esta observação, bastante incomum, mostrou que também outros fungos basidiomicetos podem fazer parte das estratégias de forrageamento dessas formigas.

Os fatos estão mostrando que a simbiose das formigas Attini, atualmente aceito como sendo constituído por fungo basidiomiceto mutualista - fungos filamentosos – leveduras - bactérias e actinobactérias, não é um modelo simples de se estudar. Temos na verdade um verdadeiro micro-ecosistema, onde algumas espécies, ao menos à luz do conhecimento atual, se mostram como “micoparasitas”, outras como “oportunistas”, outras como “mutualistas” e, a grande maioria ainda não revelou o papel que podem desempenhar no “organismo – formigueiro”.

Os trabalhos desenvolvidos com as formigas basais coletadas no Campus da UNESP-Rio Claro e pertencentes ao gênero *Mycocepurus*, confirmaram nossa afirmativa acima, pois mostraram uma vez mais o pouco que realmente se conhece sobre estas formigas. Apresentamos a descrição de uma espécie nova de um micoparasita específico, gênero comumente descrito como associado aos jardins de fungos das formigas derivadas ou “higher Attini”. Por outro lado, a presença de estruturas idênticas a verdadeiros gongilídeos

produzidos pelo fungo da formiga basal *M. smithii* também modifica o cenário atual, pois esta característica é tratada na literatura como um marco evolutivo único das formigas derivadas ou “higher Attini”.

Esperamos poder dar continuidade futuramente a algumas pesquisas que começamos nesta etapa e quem sabe possamos vir a contribuir com algum entendimento maior sobre as inter-relações dos micro-organismos entre sí e com as formigas.

APÊNDICE A