Saudi Journal of Biological Sciences (2016) xxx, xxx–xxx

King Saud University

Saudi Journal of Biological Sciences

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Isolation of the symbiotic fungus of *Acromyrmex pubescens* and phylogeny of *Leucoagaricus gongylophorus* from leaf-cutting ants

G.A. Bich [a,b,*], M.L. Castrillo [a,b], L.L. Villalba [b], P.D. Zapata [b]

[a] *Microbiology and Immunology Laboratory, Misiones National University, 1375, Mariano Moreno Ave., 3300 Posadas, Misiones, Argentina*
[b] *Biotechnology Institute of Misiones "María Ebe Reca", 12 Road, km 7, 5, 3300 Misiones, Argentina*

Received 7 August 2015; revised 21 April 2016; accepted 10 May 2016

KEYWORDS

ITS;
Leaf-cutting ants;
Leucoagaricus;
Phylogeny

Abstract Leaf-cutting ants live in an obligate symbiosis with a *Leucoagaricus* species, a basidiomycete that serves as a food source to the larvae and queen. The aim of this work was to isolate, identify and complete the phylogenetic study of *Leucoagaricus gongylophorus* species of *Acromyrmex pubescens*. Macroscopic and microscopic features were used to identify the fungal symbiont of the ants. The ITS1-5.8S-ITS2 region was used as molecular marker for the molecular identification and to evaluate the phylogeny within the *Leucoagaricus* genus. One fungal symbiont associated with *A. pubescens* was isolated and identified as *L. gongylophorus*. The phylogeny of *Leucoagaricus* obtained using the ITS molecular marker revealed three well established monophyletic groups. It was possible to recognize one clade of *Leucoagaricus* associated with phylogenetically derived leaf-cutting ants (*Acromyrmex* and *Atta*). A second clade of free living forms of *Leucoagaricus* (non-cultivated), and a third clade of *Leucoagaricus* associated with phylogenetically basal genera of ants were also recognized. The clades corresponded to traditional taxonomic groups, and were differentiated by ecological habitats of different species.

1. Introduction

There is an ancient, highly evolved, mutualism between fungus-growing ants (Hymenoptera: Formicidae: Attini) and their fungi (Agaricales) (Currie, 2001; Wetterer et al., 1998; Wilson, 1971).

The ability to cultivate fungi occurs just in a few groups of ants, and all these ants belong to the Attini tribe which is composed of 12 genera and approximately 210 species (Schultz and Meier, 1995). Most of these genera of ants use dead matter to

* Corresponding author at: Misiones National University, 1375, Mariano Moreno Ave., 3300 Posadas, Misiones, Argentina. Tel.: +54 (0376) 4435118; fax: +54 (0376) 4425414.
E-mail address: gustavobich@gmail.com (G.A. Bich).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2016.05.010
1319-562X © 2016 The Authors. Published by Elsevier B.V. on behalf of King Saud University

cultivate their fungal symbiont except the leaf-cutting ants. The latter ants use fresh plant materials for manuring their gardens and belong to the *Atta* and *Acromyrmex* genera (Currie, 2001).

Atta and *Acromyrmex* genera of ants are the most serious agricultural pest of tropical and subtropical America, causing enormous economic damage to the neotropic agricultural industry (Wirth et al., 2003). The possibility of controlling leaf-cutting ants with biological methods has generated great interest in diverse studies involving the fungi cultivated by ants.

Ants of the Attini tribe live in obligate symbiosis with a basidiomycete fungus of the Agaricaceae family which is the essential food source for the larvae and queen (Currie, 2001; Fisher et al., 1994; Lange and Grell, 2014; Miyashira et al., 2010). On the other hand, the ants provide the fungal symbiont with a variety of substrates and also stimulate the fungal growth (Currie, 2001; Lugo et al., 2013; North et al., 1997).

The Agaricales order is one of the most diverse and largest orders of the Basidiomycota Phylum (Misra et al., 2012). The family Agaricaceae is a widely distributed group of saprotrophic fungi and many taxa exist in this family which makes the taxonomical identification quite complex (Misra et al., 2012; Moncalvo et al., 2000; Vellinga et al., 2003, 2010). To date, according to Kirk et al. (2008), this family comprises 85 genera and 1340 species.

Several authors reported the fungal symbiont of the fungus growing ants as *Leucoagaricus gongylophorus* species (Fisher et al., 1994; Miyashira et al., 2010; Silva et al., 2004). According to the literature *Leucoagaricus* species belong to the family Agaricaceae and *Leucoagaricus* genus has approximately 75 species (Ortiz et al., 2008).

An understanding of mutualism between the fungal symbiont and the Attini ants is greatly hindered by the lack of information regarding the taxonomic placement and evolutionary history of the fungal cultivars (Currie, 2001). The taxonomy and hence the diversity of the symbiotic fungi of ants have not yet been completely elucidated (Silva et al., 2004).

The traditional methods for fungal taxonomy and systematics depend on the morphology of the fruiting body. Ferreira and Cortez (2012) described some macromycetes belonging to the *Leucoagaricus* genus in the region. Since the fungi cultivated by Attini ants do not actually produce these sexual structures in association with the ants or in pure culture, their identification becomes quite complex (Fisher et al., 1994; Hervey et al., 1977; Lugo et al., 2013; North et al., 1997).

Other methods for fungal taxonomy and systematics use molecular markers. Nowadays, the systematic relationships of fungi have been greatly assisted by new evidence from molecular systematics, mostly using ribosomal DNA sequence data (Schoch et al., 2012; White et al., 1990).

Moncalvo et al. (2002) conducted a molecular phylogenetic study of Euagarics with nuclear large ribosomal subunit gene sequences (nLSU) and although they demonstrated the existence of several clades composed of members of diverse traditional groups, they placed most *Leucoagaricus* species and fungal symbionts of leaf-cutting ants in one single clade.

In some studies, genetic markers such as ribosomal gene (rDNA), internal transcribed spacer (ITS), and part of elongation factor 1-α gene (EF 1-α), have been used for the molecular characterization and phylogenetic studies of fungal symbionts of ants (Chapela et al., 1994; Lugo et al., 2013; Ortiz et al., 2008; Silva et al., 2004).

In Argentina there is only one initial molecular study of the *Leucoagaricus* fungus (Lugo et al., 2013). These authors isolated and identified both, morphologically and genetically the fungi cultured by two populations of *Acromyrmex lobicornis* from the center of Argentina. But studies on morphological and molecular characterization of leaf-cutting ant symbionts isolated from other regions of Argentina still remain to be done. Therefore, the contribution of species inhabiting in Misiones (located in the North of Argentina) will allow further understanding on new populations.

Currently there are several hypotheses about the phylogenetic origin of fungi associated with Attini ants. Accordingly, nowadays phylogenetic relationship studies about ant's fungal symbiont are under extensive discussion (Bot et al., 2001; Chapela et al., 1994; Silva et al., 2004).

The objectives of our study were: (1) to determine the taxonomic identity of one *Acromyrmex pubescens* fungal symbiont strain using morphological and molecular characterization; and (2) to study the phylogenetic relationships among the fungal symbiont strain and related species from the *Leucoagaricus* genus.

2. Materials and methods

2.1. Fungal isolate and culture conditions

Portions of fungal garden were collected from nineteen nests of *Acromyrmex* ants from Misiones province, in the North of Argentina. Dextrose Potato Agar and Malt Extract Agar LP Media were used for the isolation of the mycelial form of the fungus (Miyashira et al., 2010) (Collection date: September 2014. Collectors/isolators: Castrillo and Bich). The fungal garden portions were subcultured periodically until pure isolates were attained. The fungus garden material was incubated in the dark at 28 °C and 80% humidity for at least 20 days. The isolate was deposited in culture collection of the Universidad Nacional de Misiones with accession number LBM 190.

2.2. Morphological identification of Leucoagaricus isolates

After a 5–10-day period, the macroscopic characteristics of each fungal colony developed from the fungus garden material were described through the observation of the following parameters: growth rate, aspect and colonial color upside and reverse.

Microscopic features like presence/absence of septum, mycelia size, basidia or other structures, etc. were observed for morphological identification. *Leucoagaricus* genus typically presents a special mycelial structure called gongylidia and the absence of fibula (Currie, 2001; Miyashira et al., 2010).

2.3. DNA extraction

Approximately 200 mg mycelium was placed in a 1.5-mL microcentrifuge tube. The mycelium was disrupted with a glass pestle that fitted inside the tube with DNA extraction Buffer (Tris EDTA buffer pH 8, containing 1.5 M sodium chloride, Proteinase K 0.1 mg/mL, β-mercaptoethanol 10 mM and

Please cite this article in press as: Bich, G.A. et al., Isolation of the symbiotic fungus of *Acromyrmex pubescens* and phylogeny of *Leucoagaricus gongylophorus* from leaf-cutting ants. Saudi Journal of Biological Sciences (2016), http://dx.doi.org/10.1016/j.sjbs.2016.05.010

SDS 2% (w/v). The nucleic acids were purified using a chloroform:isoamyl alcohol mixture (24:1, v/v). Then nucleic acids were precipitated with ethanol and potassium acetate, washed with ethanol, air-dried, and finally dissolved in DNAse-free water (Fonseca, 2012).

2.4. Amplification of ribosomal DNA and internal transcribed spacer regions

For the molecular identification and phylogeny analysis the ITS1-5.8S-ITS2 region was evaluated. PCR amplifications were carried out in a 20-μL reaction mixture which included 1× PCR Buffer, 2.5 mM $MgCl_2$, 200 μM of dNTP mix, 10 pmol of each of the amplification primers, 0.5 U of Taq polymerase (InBio), and 5–20 ng genomic DNA (Fonseca, 2012). The primers used were the ITS1 F-(5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 R-(5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The amplification protocol consisted of an initial denaturation at 94 °C for 4 min, followed by 35 PCR amplification cycles of 94 °C for 40 s, 53 °C for 40 s and 72 °C for 40 s. A final extension step of 72 °C for 10 min was included. The amplified fragment was stained and evaluated in agarose gel electrophoresis. Both strands of PCR products were sequenced by Macrogen Korea for further phylogenetic studies.

2.5. Sequence analysis

The ITS1-5.8S-ITS2 sequence generated in this study was deposited in GenBank under accession number KJ784481. The ITS sequence of the fungal strain was compared with sequences in GenBank and Fungal Barcoding data bank sites.

Also, approximately 70 accessions of the ITS1-5.8S-ITS2 sequences were selected and retrieved from the GenBank data base (National Center for Biotechnology Information) representing most species within the *Leucoagaricus* genus with available molecular data. Nucleotide sequences retrieved in this study consist of about 600 bp which correspond to the ITS1-5.8S-ITS2 regions. Sequences of *L. gongylophorus* from leaf-cutting ants (*Atta* and *Acromyrmex*) and from fungus-growing ants, other than leaf-cutting ants, also were retrieved from GenBank for the study. In addition, sequences from free living forms of *Leucoagaricus* were retrieved. A sequence from the Ascomycota *Beauveria bassiana* was used to root the *Leucoagaricus* phylogenetic tree. The DNA sequences were aligned using the Clustal W program (Thompson et al., 1997). The phylogenetic methods were based on one distance-based method (Neighbor Joining – NJ) and one cladistic method (Maximum Parsimony – MP). For the phylogenetic tree evaluation the Bootstrap analyses were conducted based on 1000 replicates. The MEGA 6.0 (Tamura et al., 2013) package was used for the analyses. Phylogenetic data have been submitted to TreeBase with submission number S17841 (http://purl.org/phylo/treebase/phylows/study/TB2:S17841).

3. Results

To isolate the symbiotic fungi of leaf-cutting ants from the North of Argentina, nineteen nests of *Acromyrmex* ants were sampled from the province of Misiones. After incubating different portions of the fungal garden from the nests, fungal colonies were observed to develop.

From all the colonies observed it was possible to isolate just one strain of *Leucoagaricus* free of contaminants from one *A. pubescens* nest. This fungal isolate was preserved in culture collection of the Universidad Nacional de Misiones under the accession number LBM 190 for further studies.

White to cream-colored colonies with slow growth were subcultured for the isolation of the symbiotic fungi, followed by the microscopic identification of the putative *Leucoagaricus* colonies (Fig. 1).

At the optical microscope these fungal colonies presented hyaline hyphae without fibula. The hyphae also presented swollen tips (40 μm × 30 μm). The observation by optical microscopy of gongylidia on mycelia fragments together with macroscopic colonial characters supported the morphological identification of the mutualistic fungus as *Leucoagaricus* sp. (Fig. 2).

From the latter fungal isolate it was possible to obtain good quality DNA that is used for the amplification and sequencing to confirm the identification of the *Leucoagaricus* isolate. The DNA amplification produced a fragment of approximately 600 bp comprising the ITS1 region, the 5.8S gene and the ITS2 region.

This ITS1-5.8S-ITS2 sequence with other selected sequences from GenBank were aligned and used to construct the phylogenetic tree of *Leucoagaricus*. The ITS1-5.8S-ITS2 sequence generated in this study was deposited in GenBank under the accession number KJ784481.

The sequences were analyzed separately by Neighbor Joining (NJ) and Maximum Parsimony (MP) analyses, and the resulting trees were compared (Fig. 3). No divergences were detected between phylogenies, thus indicating that the datasets could be combined. Both NJ and MP analyses were useful for discriminating *Leucoagaricus* isolates at the species level.

Analysis of the ITS1-5.8S-ITS2 sequences of different *Leucoagaricus* placed fungal species in three different clades. All *L. gongylophorus* strains from leaf-cutting ants were located in a monophyletic group (bootstrap support of 100%). The sequence of our fungal isolate was included in CLADE 1.

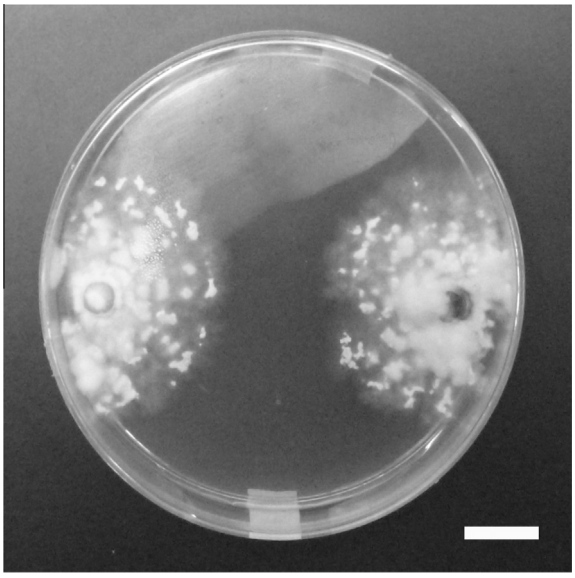

Figure 1 *Leucoagaricus gongylophorus* from *Acromyrmex pubescens* nest. Macroscopic view of the colony growth in Petri dish. (Bar = 1.5 cm).

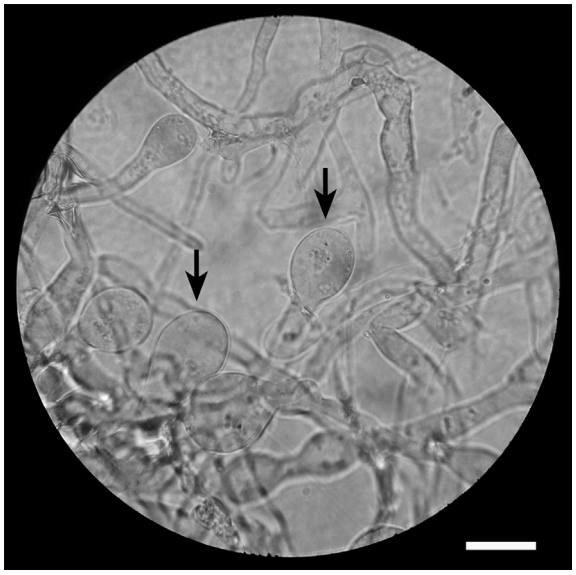

Figure 2 Microscopic mycelia detail from mutualistic fungi. Enlarged apical structures correspond to gongylidia (arrows). (Bar = 40 μm).

The CLADE 1 of *L. gongylophorus* strains from leaf-cutting ants was more closely related to free living forms of *Leucoagaricus* strains (CLADE 2) than to the CLADE 3 of *L. gongylophorus* from other fungus-growing ants (non *Atta* or *Acromyrmex* ants).

The phylogenetic analyses confirmed the taxonomic identity of the *Leucoagaricus* isolate from *A. pubescens* nest from the North of Argentina as *L. gongylophorus*. The isolate obtained in this study is phylogenetically indistinguishable from *L. gongylophorus* isolated from other genera of leaf-cutting ants belonging to the genera *Atta* and *Acromyrmex*, at least using this particular molecular marker.

Also according with this molecular marker, all isolates of *L. gongylophorus* from leaf-cutting ants had little genetic distance among them, but were clearly distinguished from other strains of *L. gongylophorus* grown by lower fungus-growing ants (CLADE 3). Moreover, isolates of *L. gongylophorus* from leaf-cutting ants were genetically distinguished from other species of free living forms of *Leucoagaricus*.

4. Discussion

Gongylidia are typical structures of the symbiotic fungi of higher attine ants (Chapela et al., 1994; Silva et al., 2004). These structures were recognized in the isolated fungus in our study. Miyashira et al. (2010) and Lugo et al. (2013) completed the morphologic identification of the symbiont fungus of *Atta* and *Acromyrmex* leaf-cutting ants using macro- and microscopic characters. In our study, the morphologic identification of the fungus symbiont isolate from one *A. pubescens* nest was consistent with current taxonomic identification of *L. gongylophorus*.

Molecular methods represent a more sensitive and rapid strategy than morphological techniques to identify species, also molecular identification strategies eliminate diverse technical limitations (Chakraborty et al., 2011). Molecular methods do not replace morphological characterization, conversely they complement morphological methods. ITS region is typically useful for molecular systematics of species (Baldwin, 1992; Schmidt and Moreth, 2002; Schoch et al., 2012), because ITS sequences can accumulate mutations at a faster rate than the 5.8S, 18S, and 28S rRNA genes.

The phylogeny of *Leucoagaricus* genus was reconstructed by employing the ITS sequence from our isolate and from data of the GenBank. The phylogeny is depicted in Fig. 3.

With respect to their taxonomic and systematic location, the genus *Leucoagaricus* is controversial; Lugo et al. (2013) analyzed the phylogeny of *L. gongylophorus* with ITS sequences from diverse species of *Atta* and *Acromyrmex* and demonstrated that the fungus grown in ant nests is a unique species. The latter findings also support the results found in our study.

Pereira et al. (2015) made a Phylogenetic analysis of the ITS region where the tree showed two distinct groups (branches in tree) regarding the symbiont fungus isolates from *Acromyrmex heyeri* and *Acromyrmex ambiguous* evidencing differences in the strain. So in that study the authors proposed that each of the latter leaf-cutting ant species cultivate the same *L. gongylophorus* species but their own strain. Those findings were in contrast with a part of our results, because our Phylogenetic tree using more taxa (*L. gongylophorus* from more leaf-cutting ants and from more regions) made a unique clade.

Otherwise, based on phylogenetic evidence the strains of *L. gongylophorus* that occur in the phylogenetically basal genera of ants (i.e. *Trachymyrmex* and *Sericomyrmex*) formed a distinct clade from those that occur in the phylogenetically derived leaf-cutting ants (i.e. *Acromyrmex* and *Atta*). This clade could be explained by the hypothesis that those strains of *L. gongylophorus* were recently domesticated from free-living populations of Agaricaceae (Mueller et al., 1998). Vo et al. (2009) also found that fungi of lower attine ants have close free-living relatives.

Chapela et al. (1994) worked with the phylogeny of the nuclear 28S ribosomal DNA of the fungal symbiont and found a similar phylogenetic tree, with a clade of fungi from derived higher attine ants and another from phylogenetically basal ants.

All strains of *L. gongylophorus* from *Atta* and *Acromyrmex* from diverse regions of South America were grouped together with low genetic variability. Interestingly the phylogenetic tree couldn't separate *L. gongylophorus* strains from *Atta* from those *L. gongylophorus* strains isolated from *Acromyrmex*. The fact that strains of *L. gongylophorus* of the higher Attini ants made a sole clade could indicate the conservation of the phylogenetic relationship of these strains of fungal symbiont. Generally it had been proposed that these fungal strains are clonally propagated by their ant hosts (Chapela et al., 1994). One noteworthy example of the evolutionary cost to cultivar clonality is a slower evolutionary response to parasites (Currie et al., 1999).

In the phylogeny depicted in Fig. 3, the free-living *Leucoagaricus* species (non-cultivated) formed a single clade isolated from the strains cultivated by ants. In this study the observed clades are only composed of either cultivated symbiont (CLADE 1 and 3) or saprophytic taxa (CLADE 2).

The existence of these three defined clades, composed of either cultivated symbiont or saprophytic strains, may indicate that these two ecological habitats are well established in fungi. Moncalvo et al. (2002), in their study on Euagarics, reported that the association between fungi of Agaricaceae family and

Please cite this article in press as: Bich, G.A. et al., Isolation of the symbiotic fungus of *Acromyrmex pubescens* and phylogeny of *Leucoagaricus gongylophorus* from leaf-cutting ants. Saudi Journal of Biological Sciences (2016), http://dx.doi.org/10.1016/j.sjbs.2016.05.010

Figure 3 Phylogenetic tree from Neighbor Joining analyses showing the phylogenetic relationships in the *Leucoagaricus* genus. Neighbor Joining tree was performed using the ITS1-5.8S-ITS2 sequences. Sequence of our *Leucoagaricus gongylophorus* strain is indicated in bold letters. Numbers on branches correspond to bootstrap values obtained with 1000 replicates. GenBank accession numbers of the sequences used in this study are indicated after each *Leucoagaricus* name.

insects had been developed phylogenetically in an independent way several times. These authors proposed that these associations appear to be evolutionarily stable because the lack of observed reversal to a free-living habit of fungi.

From these results it can be concluded that the fungus isolated from the *A. pubescens* nest belongs to the *L. gongylophorus* species based on macroscopic, microscopic and molecular data.

Also, from the phylogeny analysis, using the ITS molecular marker, a total of three *Leucoagaricus* monophyletic groups (clades) could be recognized. The clades corresponded to traditional taxonomic groups, and these clades were also supported by ecological habits of the strains. *L. gongylophorus* sequences of the phylogenetically basal genera of ants (i.e. *Trachymyrmex* and *Sericomyrmex*) were grouped in a distinct clade from those sequences from the phylogenetically derived leaf-cutting

Please cite this article in press as: Bich, G.A. et al., Isolation of the symbiotic fungus of *Acromyrmex pubescens* and phylogeny of *Leucoagaricus gongylophorus* from leaf-cutting ants. Saudi Journal of Biological Sciences (2016), http://dx.doi.org/10.1016/j.sjbs.2016.05.010

ants (*Acromyrmex* and *Atta*). Also *L. gongylophorus* species of the leaf-cutting ants were phylogenetically conserved. The free living forms of *Leucoagaricus* (non-cultivated) made a sole clade, isolated from the strains cultivated by ants. This is the first study of the isolation, identification and phylogeny of *L. gongylophorus* species of *Acromyrmex* in the North of Argentina.

Acknowledgements

This work has been carried out with support from Misiones National University (16Q524-year 2013–2014). Castrillo is a postgraduate CONICET – Argentina fellowship holder and Bich is a postgraduate CONICET-CEDIT Misiones (Argentina) fellowship holder.

References

Baldwin, B., 1992. Phylogenetic utility of the internal transcribed spacers for nuclear ribosomal DNA in plants: an example from the *Compositae*. Mol. Phylogenet. Evol. 1, 3–16.

Bot, A.N., Rehner, S.A., Boomsma, J.J., 2001. Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. Evolution 55, 1980–2001.

Chakraborty, B.N., Chakraborty, U., Dey, P.L., Rai, K., 2011. RDNA sequence and phylogenetic analysis of *Macrophomina phaseolina*, root rot pathogen of *Citrus reticulata* (Blanco). Global J. Mol. Sci. 6, 26–34.

Chapela, I.H., Rehner, S.A., Schultz, T.R., Mueller, U.G., 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266, 1691–1694.

Currie, C.R., 2001. Community of ants, fungi and bacteria: a multilateral approach to studying symbiosis. Annu. Rev. Microbiol. 55, 357–380.

Currie, C.R., Mueller, U.G., Malloch, D., 1999. The agricultural pathology of ant fungus gardens. Proc. Natl. Acad. Sci. U.S.A. 96, 7998–8002.

Ferreira, A.J., Cortez, V.G., 2012. Lepiotoid *Agaricaceae* (*Basidiomycota*) from São Camilo State Park, Paraná State, Brazil. Mycosphere 3, 962–976.

Fisher, P.J., Stradling, D.J., Pegler, D.N., 1994. *Leucoagaricus* basidiomata from a live nest of the leaf-cutting ant *Atta cephalotes*. Mycol. Res. 98, 884–888.

Fonseca, M.I., 2012. Utilización de hongos de pudrición blanca de la provincia de Misiones en procesos de biopulpado: Aspectos bioquímicos y moleculares de sistemas ligninolíticos involucrados y prospección biotecnológica (Tesis para optar al grado de Doctor en Ciencias de Recursos Naturales). Univ. Nac. Tucumán. Fac. Bqca, Qca y Fcia, p. 200.

Hervey, A., Rogerson, C.T., Leong, I., 1977. Studies on fungi cultivated by ants. Brittonia 2, 226–236.

Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A., 2008. Ainsworth & Bisby's Dictionary of the Fungi, 10th ed. CAB International, Wallingford.

Lange, L., Grell, M.N., 2014. The prominent role of fungi and fungal enzymes in the ant–fungus biomass conversion symbiosis. Appl. Microbiol. Biotechnol. J. 98, 4839–4851.

Lugo, M.A., Crespo, E.M., Cafaro, M., Jofré, L., 2013. Hongos asociados con dos poblaciones de *Acromyrmex lobicornis* (Formicidae) de San Luis, Argentina. Bol. Soc. Argent. Bot. 48, 5–15.

Misra, J.K., Tewari, J.P., Deshmukh, S.K., 2012. Systematic and Evolution of Fungi. Taylor & Francis Group, USA.

Miyashira, C.H., Tanigushi, D.G., Gugliota, A.M., Santos, D.Y., 2010. Comparison of radial growth rate of the mutualistic fungus of *Atta sexdens rubropilosa* Forel in two culture media. Braz. J. Microbiol. 41, 506–511.

Moncalvo, J.M., Lutzoni, F., Rehner, S., Johnson, J., Vilgalys, R., 2000. Phylogenetic relationships of Agaric fungi based on nuclear large subunit ribosomal DNA sequences. Syst. Biol. 49, 278–305.

Moncalvo, J., Vilgalys, R., Redhead, S., Johnson, J., James, T., Aime, C., Hofstetter, V., Verduin, S., Larsson, E., Baroni, T., Thorn, G., Jacobsson, S., Clemencon, H., Miller Jr., O., 2002. One hundred and seventeen clades of euagarics. Mol. Phylogenet. Evol. 23, 357–400.

Mueller, U.G., Rehner, S.A., Schlutz, T.R., 1998. The evolution of agriculture in ants. Science 281, 2034–2038.

North, R.D., Jackson, C.W., Howse, P.E., 1997. Evolutionary aspects of ant-fungus interactions in leaf-cutting ants. Trends Ecol. Evol. 10, 386–389.

Ortiz, A., Franco-Molano, A.E., Bacci, M., 2008. A new species of *Leucoagaricus* (Agaricaceae) from Colombia. Mycotaxon 106, 371–378.

Pereira, D.I., Botton, S., Azevedo, M.I., Monteiro, D., Weiblen, C., Machado, V., Donatti, M., Loeck, A., 2015. Isolation and molecular characterization of symbiotic fungus from *Acromyrmex ambiguus* and *Acromyrmex heyeri* ants of Rio Grande do Sul State, Brazil. Ciênia Rural 7, 1256–1261.

Schmidt, O., Moreth, U., 2002. Data bank of rDNA-ITS sequences from building-rot fungi for their identification. Wood Sci. Technol. 36, 429–433.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Fungal Barcoding Consortium, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. U.S.A. 109, 6241–6246.

Schultz, T.R., Meier, R., 1995. A phylogenetic analysis of the fungus-growing ants (Hymenoptera: Formicidae: Attini) based on morphological characters of the larvae. Syst. Entomol. 20, 337–370.

Silva, A.C., Bacci, M.J., Hinkle, M.L., Sogin, M.L., Pagnocca, F.C., Martins, V.G., Bueno, O.C., Hebling, M.J., 2004. Low variation in ribosomal DNA and internal transcribed spacers of the symbiotic fungi of leaf-cutting ants (Attini: Formicidae). Braz. J. Med. Biol. Res. 37, 1463–1472.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F., Higgins, D., 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4872–4882.

Vellinga, E.C., Kok, R.P., Bruns, T.D., 2003. Phylogeny and taxonomy of Macrolepiota (Agaricaceae). Mycologia 95, 442–456.

Vellinga, E.C., Contu, M., Vizzini, A., 2010. *Leucoagaricus decipiens* and *La. erythrophaeus*, a new species pair in sect. Piloselli. Mycologia 102, 447–454.

Vo, T.L., Mueller, U., Mikheyev, A., 2009. Free-living fungal symbionts (Lepiotaceae) of fungus-growing ants (Attini: Formicidae). Mycologia 101, 206–210.

Wetterer, J.K., Schultz, T.R., Meier, R., 1998. Phylogeny of fungus-growing ants (tribe attini) based on mtDNA sequence and morphology. Mol. Phylogenet. Evol. 1, 42–47.

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

Wilson, E.O., 1971. The Insect Societies. Belknap, Cambridge, MA.

Wirth, R., Beyschlag, W., Ryel, R., Herz, H., Hölldobler, B., 2003. The herbivory of leaf-cutting ants. A case study on Atta colombica in the tropical rainforest of Panama. In: Ecological Studies Series, vol. 164. Springer Verlag Berlin, Heidelberg, New York, p. 230.