Scientia Fungorum vol. **46**: 3-8 **2017**

In vitro isolation and identification of *Leucoagaricus gongylophorus* from *Atta mexicana* (Hymenoptera: Formicidae) fungal garden

Aislamiento *in vitro* e identificación de *Leucoagaricus gongylophorus* de un jardín de hongos de *Atta mexicana* (Hymenoptera:Formicidae)

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Abstract

Background: The leaf-cutter ant species (*Atta* and *Acromirmex*) have a mutualistic relationship with the basidiomycete fungus *Leucoagaricus gongylophorus* (Agaricaceae). This relationship is crucial to the life cycles of both organisms.
Objectives: Due to the lack of reports about isolation of the fungus cultivated by the ant *Atta mexicana* (Formicidae), the objectives of this work were *in vitro* isolation and identification of *L. gongylophorus* from the fungal garden of *A. mexicana*.
Methods: The identification of the fungi was carried out by observation of macroscopic and microscopic characteristics, as well as molecular and phylogenetic analysis based on ITS region of rDNA genes' cluster.
Results and conclusions: The phylogenetic analysis demonstrated 99% identity with the ITS sequences of *L. gongylophorus* presented in GenBank that were reported for other fungal isolates from Attini tribe nests developed by genera *Atta* and *Acromyrmex*. For this reason, this work is the first report of *in vitro* isolation and identification of *L. gongylophorus* from the nest of *Atta mexicana*.

Key words: *In vitro* cultivation, fungal symbiont, Gongylidias.

Resumen

Antecedentes: Las especies de hormigas cortadoras de hojas (*Atta* y *Acromirmex*) presentan una relación mutualista con el hongo basidiomycete *Leucoagaricus gongylophorus*. Esta relación es crucial para los ciclos de vida tanto de las hormigas como para el hongo.
Objetivos: Debido a que no existen reportes sobre el aislamiento *in vitro* del hongo cultivado por la hormiga *Atta mexicana*, este trabajo tuvo como objetivo el aislamiento *in vitro* e identificación de *L. gongylophorus* a partir del jardín de hongo de la hormiga *A. mexicana*.
Métodos: La identificación del hongo cultivado por la hormiga se llevó a cabo mediante la observación de las características macro y microscópicas, así como un análisis molecular y filogenético usando la región ITS del ADNr.
Resultados y conclusión: El ADN aislado presentó un 99% de identidad con lo reportado para *L. gongylophorus* y es comparable con otros aislamientos fúngicos de los nidos de otras especies de la tribu Attini como *Atta* y *Acromyrmex*. Debido a ello, este trabajo es el primer reporte del aislamiento *in vitro* e identificación de *L. gongylophorus* a partir de nidos de *A. mexicana*.

Palabras clave: Cultivo *in vitro*, Simbionte fúngico, Gongilidias.

Recibido / Received: **13/02/2017**
Aceptado / Accepted: **18/12/2017**

INTRODUCTION

Fungi are not only important to human society; they also develop close relationships with other organisms (Moreira-Arana *et al.,* 2007). In this sense, mutualistic associations benefit both organisms in such a way that they are usually indispensable or necessary for their survival (Herrera and Ulloa 1998). Therefore, many fungi with symbiotic relationships with plants, algae, cyanobacteria and animals have been reported (Campbell and Reece*,* 2007). The most widespread and well-characterized symbiotic associations of fungi are with algae in lichens symbiosis) and with vascular plants in mycorrhizae. In regard to the symbiosis or mutualistic relations with animals it can be mentioned anaerobic fungi of the phylum Chytridiomycota in the rumen of herbivores (Ho and Barr, 1995), truffles with rodents (Frank *et al*., 2006) and associations of fungi with insects from orders Coleoptera, Homoptera, Hymenoptera and Isoptera. The latter associations could be explained by the fact that many insects and fungi share their habitat (Herrera and Ulloa, 1998). A good example of this kind of relationship is the one between the Basidiomycete fungus of the Agaricaceae family, *Leucoagaricus gongylophorus* (A. Møller) Singer and leaf-cutter ants of the genus *Atta* (Silva-Pinhati *et al*., 2004). This association is crucial for both organisms' life cycles, due to: (a) the fact that *L. gongylophorus* lost the ability to produce sexual spores (Stevens, 1983), and it had been probably asexually cultivated by ants for over than 23 million years (Chapela *et al*., 1994); (b) without *L. gongylophorus* as a food source of the colony, the leaf-cutter ants will die (Fisher *et al*., 1996). However, the sexual structures of *L. gongylophorus* were also reported (Fisher *et al*.*,* 1994; Pagnocca *et al*., 2001; Mueller *et al*., 2001), indicating on sexual reproduction possibility (Doherty *et al*., 2003).

The understanding of this mutual association between the fungus and the ant is not clear mainly due to the lack of taxonomic information and the evolutionary history of the cultivated mushrooms species (Currie, 2001). The main difficulty has been related with reluctance of the fungus *L. gongylophorus* to produce basidiomes, which are structures required for conventional taxonomic identification (Loeck *et al*., 2004). The ants species belong exclusively to Attini tribes (Myrmicinae), which cultivate fungi in their nests and their diet is almost completely dependent on mycelia (Herrera-Salazar, 2009). The cultivated fungus cannot survive without the ants caring the fungal garden, and reciprocally the ants need the fungus to survive (Van-Bael *et al*., 2011). This ancient mutualism is based on the hygienic behavior of the ants that protects the fungal garden from bacteria and mycoparasites, by the application of antimicrobial substances secreted by them, which eliminates among 75 and 90 percent of the microorganisms (Fernández-Marín and Wcislo, 2010). Due to that there are no reports about the *in vitro* isolation of the fungus cultivated by *Atta mexicana*, this work is focused in the *in vitro* isolation and identification of *Leucoagaricus gongylophorus* from a fungal garden of *Atta mexicana*.

MATERIALS AND METHODS

Biological material

The fungal garden of *A. mexicana* ant was prepared at Instituto de Ecología, A.C. of Mexico (INECOL). The ant and the fungus were kept in artificial terrarium (nests), adapted to their survival and reproduction conditions at temperature of 25 ± 2 °C with white light, except for the fungal garden area that was kept in darkness. Three of twelve nests were randomly chosen, and were used as a source for the fungus. Mycelium fragments and some ants were taken from each of the selected nests and transferred to previously sterilized jars for their transportation to the Laboratorio de Alta Tecnología de Xalapa (LATEX) where the isolation and purification of *Leucoagaricus gongylophorus* was carried out.

Culture medium

For *in vitro* isolation of *Leucoagaricus gongylophorus* from the fungal garden of *A. mexicana*, first, the growth was induced in a moisture plate and then subsequently on malt extract agar (MEA, DIBICO) and potato dextrose agar (PDA, DIBICO) medium. From three fungal gardens, small fragments of the mycelium were taken and disinfected with 4% sodium hypochlorite solution and 70% ethanol for 3 minutes. Each disinfected fragment was inoculated in moisture plates as well as in MEA and PDA in 5 replicas and then inoculated plates were incubated at 25 ± 2 °C for 7 days. After the incubation period or until fungal colony developed, all isolates were purified by single-spore culture cloning technique.

Morphological identification

Morphological identification was carried out through the observation of gongylidias under a microscope after 21-day incubation period at 25 °C on MEA plates. Characteristic reproductive structures of this genus from the fungal garden of *Atta mexicana* were analyzed (DeFine Licht, 2014).

Molecular identification

The fungus strain was genetically identified based on ITS-rDNA nuclear sequence analysis. Isolations of genomic DNA from the mycelium were performed according to the protocol proposed by Yu *et al.* (2011) with modifications. ITS5- ITS4 primers were employed to amplify the ITS-rDNA nuclear region through direct PCR. The amplification products of the PCR were purified using Wizard kit (Pro-mega®, USA) following the manufacture instructions and were sequenced by an Applied Biosystems (model 391) sequencer at Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM). The nucleotides sequences were edited employing BioEdit v7.0.5.3 software and were compared through BLAST search with the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) to confirm the species identity. For the phylogenetic analysis, the sequences were aligned using Clustal W algorithm from the software MEGA6 (Tamura *et al*., 2013). The nucleotide alignments were tested on jModeltest (Darriba *et al*., 2013; Guindon and Gascuel 2003) to define an evolutionary model; the phylogenetic tree was constructed using Maximum Likehood (TPM3+G) Method of MEGA6 software. The analysis was performed with boostrap value of 1000.

A B C

D E F

Figure 1. A) Fungal garden from *Atta mexicana* nest. B) Group of gongylidias stained with lactophenol blue at 100X direct from the fungal garden. C) Mycelial isolate of *L. gongylophorus* in MEA. D) Presence of secretions on the edges of *L. gongylophorus* colony. E) and F) Gongylidias stained with lactophenol blue at 100X from a isolate in MEA of *L. gongylophorus*.

RESULTS

Morphological identification

From three analyzed nests (the artificial terrariums) of *A. mexicana*, three isolates of *L. gongylophorus* were obtained. *L. gongylophorus* was isolated and maintained on MEA at 25 ± 2 °C for 60 days of incubation. After the incubation time, white cottonlike aerial colonies were produced by the mycelium at the center, and young hyaline hyphae were observed towards the edge of the colony. Later, the cultures produced secretion droplets in the aerial part of the colony and, at the edge, lumpy farinaceous area was observed, that was formed mainly by gongylidias. From these cultures and from the fungal garden of *A. mexicana,* abundant gongylidias of size of 10-15 µ and 25-30 µ respectively were observed under the light field microscopy at 100X using fresh preparation stained with lactophenol blue (Figure 1).

Molecular identification

PCR product of approximately 700 bp was obtained using the primers ITS5-ITS4 for the isolate L3 of the symbiotic fungus of the ant *A. Mexicana* fungal garden. A total of 549 bp were used for the analysis of nucleotide sequences; the comparison with BLAST determined a similarity of 99% with the basidiomycete species *L. gongylophorus*. The phylogenetic analysis revealed the fungal isolate L3 within the cluster of *Leucoagaricus gongylophorus* species (Figure 2).

DISCUSSION

In vitro isolation of *Leucoagaricus gongylophorus*, as well as the study on the mutualistic relationship between ants of the genus *Atta* and the fungus, has been carried out for several species of the genus *Atta* , among them are ant species *A. cephalotes* (Mohali, 1998), *A. capiguara, A. laevigata* (Silva-Pinhati *et al.*, 2005), *A. sexdens* (Miyashira *et al.*, 2010), *A. texana* and *A. mexicana* (Sánchez-Peña, 2005), the latter only reported aspects of the fungus-growing ant symbiosis of *A. mexicana*. Nevertheless, there are no studies on *in vitro* isolation and identification of the fungus cultured by ant species *A. mexicana*. The molecular analysis placed the isolated fungus *L. gongylophorus* particularly with those reported by Pereira *et al.* (2015) and Silva-Pinhati *et al.* (2004) for symbiotic fungi of several species of *Atta* and *Acromyr-*

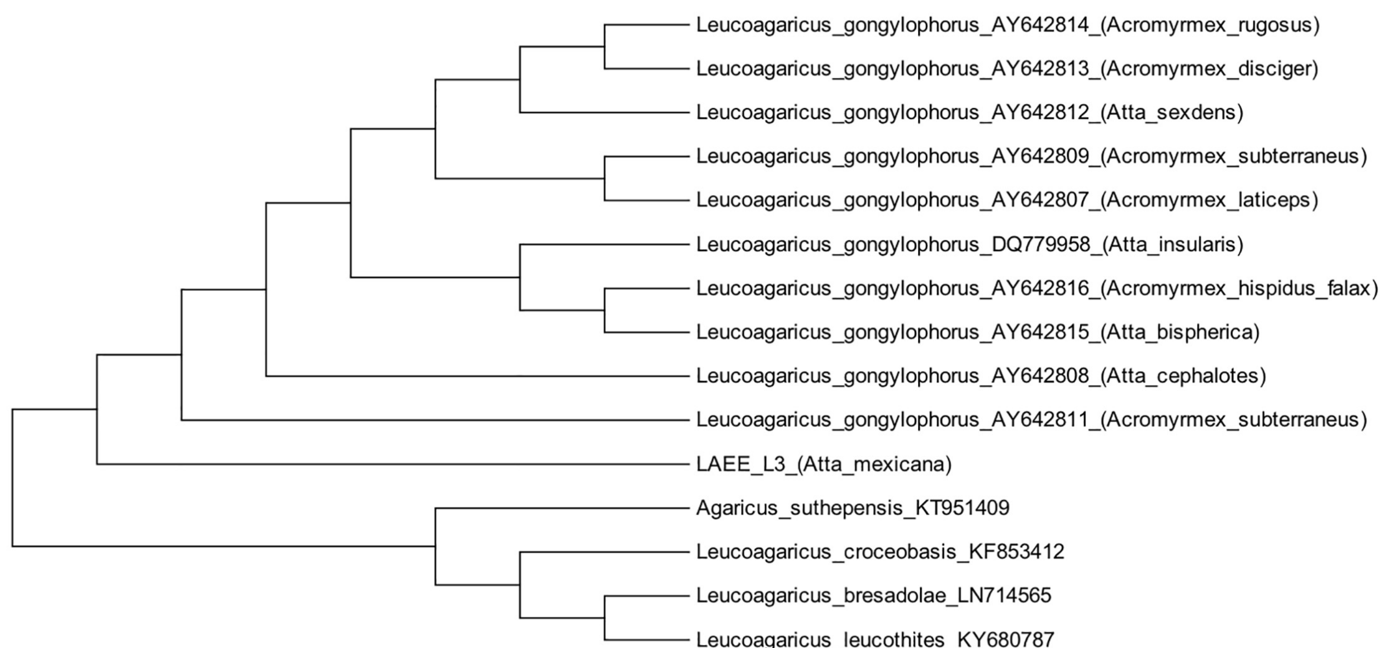

Figure 2. Phylogenetic analysis of isolate L3 of *Leucoagaricus gongylophorus* from the fungal garden of *Atta mexicana*, based in the ITS region and employing Maximum likehood with bootstrap of 1000.

mex originated from Brazil. Interestingly, *L. gongylophorus* isolates associated with the ant genera *Atta* and *Acromyrmex* were separated from other representatives of *L. gongylophorus* cultured by *Myrmicocrypta ednaella* on the dendrogram (Figure 2). Likewise, *L. gongylophorus* isolates from *Atta* and *Acromyrmex* fungal gardens were clearly separated from other species such as *L. bresoldae* and *L. croceobasis*, which were reported as free-living fungi and not cultured by ants.

Thus, our data confirm that the species *A. mexicana*, as well as other species of Attini tribe, are able to cultivate fungi of the same species *L. gongylophorus*, even when some intraspecies differences could be observed, that possibly come from co-evolution of the fungus with their respective ant species, as well as because of nutritional differences as pointed by Pereira *et al.* (2015). Additionally, it was also reported that the fungus species *L. gongylophorus* is characterized by diverse morphological traits and different physiological behavior that depends on the genus of ant that cultivates the fungus. Morphological cultural characteristics of the fungus *L. gongylophorus* are also related with the culture medium composition, pH and *in vitro* cultivating temperature (Borba *et al*., 2007).

This work reported for the first time *in vitro* isolation and identification of basidiomycete fungus *Leucoagaricus gongylophorus* from the fungal garden of *Atta mexicana* that was confirmed by employing morphological and molecular characteristics. The ant species *A. mexicana* was shown to be able to co-exist with the fungus *L. gongylophorus,* as well as the other *Atta* species such as *A. cephalotes, A. capiguara, A. laevigata, A. sexdens* and *A. texana* are also able to develop symbiotic relationship with this fungus. The aforementioned observation demonstrates that the basidiomycetes *L. gongylophorus* is the symbiotic fungus cultured by *A. mexicana ant*.

Acknowledgements

We gratefully acknowledge to the thematic network of collaboration between academic groups UV-CA-354, PRODEP 2015.

References

Borba, R.S., E.A. Loeck, J.B. Severo-Castelo, M. Marini Kopp, A.C. Oliveira, 2007. Polimorfismo do fungo simbionte de formigas cortadeiras submetido à luz ultravioleta. Ciência Rural 37: 1221-1226.

Campbell, N.A., J.B. Reece, 2007. Biología 7ª Edición. Editorial Médica Panamericana. Madrid.

Chapela, I.H., S.A. Rehner, T.R. Schultz, U.G. Mueller, 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266 (5191): 1691-1694.

Currie, C.R., 2001. A community of ants, fungi, and bacteria: a multilateral approach to studying symbiosis. Annual Review of Microbiology 55: 357-380.

Darriba D., G.L. Taboada, R. Doallo, D. Posada, 2012. Model test 2: more models, new heuristics and parallel computing. Nature Methods 9(8), 772.

De Fine Licht, H.H., J.J. Boomsma, A. Tunlid, 2014. Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nature Communications 5:5675.

Doherty, K.R., E.W. Zweifel, N.C. Elde, M.J. Mckone, S.G. Zweifel, 2003. Random amplified polymorphic DNA markers reveal genetic variation in the symbiotic fungus of leaf-cutting ants. Mycologia 95: 19-23.

Fernández-Marín, H., W.T. Wcislo, 2010. Hormigas cultivadoras de hongos. Investigación y Ciencia 409: 12-13.

Fisher, P.J., D.J. Stradling, D.N. Pegler, 1994. Leaf-cutting ants, their fungus gardens and the formation of basidiomata of *Leucoagaricus gongylophorus*. Mycologist 8: 128-131.

Fisher, P.J., D.J. Stradling, B.C. Sutton, L.E. Petrini, 1996. Microfungi in the fungus gardens of the leaf-cutting ant *Atta cephalotes*: a preliminary study. Mycological Research 100: 541-546.

Frank, J.L., S. Barry, D. Southworth, 2006. Mammal mycophagy and dispersal of mycorrhizal inoculum in Oregon white oak woodlands. Northwest Science 80: 264-273.

Guindon S., O. Gascuel, 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Systematic Biology 52: 696-704.

Herrera, T., M. Ulloa, 1998. El Reino de los hongos: Micología básica y aplicada. UNAM-Fondo de Cultura Económica. 2a Edición. México.

Herrera-Salazar, E.E., 2009. Desarrollo de una formulación granular base para el control biológico de las hormigas forrajeras (*Atta* spp.). Tesis Ms. Sc. Turrialba, CATIE, Costa Rica.

Ho, Y., D. Barr, 1995. Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from Malaysia. Mycologia 87: 655-677.

Loeck, A.E., C.R. Pierobom, L.G.D. Gusmão, A.P. Afonso, 2004. Growth of symbiont fungi of some higher attine ants in mineral medium. Ciência Rural 34: 79-82.

Miyashira, C.H., D.G. Tanigushi, A.M. Gugliotta, D.Y.A.C. Santos, 2010. Comparison of radial growth rate of the mutualistic fungus of *Atta sexdens rubropilosa* Forel in two culture media. Brazilian Journal of Microbiology 41: 506-511.

Mohali, S., 1998. Ultrastructural and morphological study of the mutualistic fungus of the ant *Atta cephalotes*. Revista de Ecología Latino Americana 5: 1-6.

Moreira-Arana, C.E., C.A. Cubillas-Ramírez, D. Trejo-Aguilar, 2007. Del “círculo de las brujas” al hongo atómico In: Zulueta-Rodríguez, R., D. Trejo-Aguilar, A.R. Trigos-Landa (eds.), El maravilloso mundo de los hongos. Universidad Veracruzana, México Pp. 31-38.

Mueller, U.G., T.R. Schultz, C.R. Currie, R.M.M. Adams, D. Malloch, 2001. The origin of attine ant-fungus mutualism. The Quarterly Review of Biology 76: 169-197.

Pagnocca F., M. Jr. Bacci, M.H. Fungaro, O.C. Bueno, M.J. Hebling, A. Sant Anna, M. Capelari, 2001. RAPD analysis of the sexual state and sterile mycelium of the fungus cultivated by the leaf-cutting ant *Acromyrmex hispidus* fallax. Mycological Research 105: 173-176.

Pereira, D.I.B., S.A. Botton, M.I. Azevedo, M.D. Urach, C. Weiblen, M.V. Schopf, D.M. Guimarães, L.A. Enimar, 2015. Isolation and molecular characterization of symbiotic fungus from *Acromyrmex ambiguus* and *Acromyrmex heyeri* ants of Rio Grande do Sul State, Brazil. Ciência Rural 45: 1256-1261.

Sánchez-Peña, S.R., 2005. Essays on organismal aspects of the fungus-growing ant symbiosis: ecology, experimental symbiont switchs and fitnes of*, Atta,* and a nex theory on the origin of ant fungiculture. Tesis de Doctorado, Universidad de Texas, EUA.

Silva-Pinhati, A.C.O., M. Bacci Jr, G. Hinkle, M.L. Sogin, F.C. Pagnocca, V.G. Martins, O.C. Bueno, M.J.A. Hebling, 2004. Low variation in ribosomal DNA and internal transcribed spacers of the symbiotic fungi of leaf-cutting ants (Attini: Formicidae). Brazilian Journal of Medical and Biological Research 37: 1463-1472.

Silva-Pinhati, A.C.O., M. Bacci Jr, C.G. Siqueira, A. Silva, F.C. Pagnocca, O.C. Bueno, M.J.A. Hebling, 2005. Isolation and maintenance of symbiotic fungi of ants in the Tribe Attini (Hymenoptera: Formicidae). Ecology, behavior and bionomics. Neotropical Entomology 34: 1-5.

Stevens, G.C., 1983. *Atta cephalotes* (zompopas, leaf-cutting ants). In: Janzen D.H. (ed.), Costa Rican Natural History. Chicago University Press. Pp. 688-691.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28: 2731-2739.

Van-Bael, S.A., C. Estrada, W.T. Wcislo, 2011. Fungal-fungal interactions in leaf-cutting ant agriculture. Psyche : Article ID 617478, 9 pages. doi:10.1155/2011/617478.

Yu, H., D. Chronis, S. Lu, X. Wang, 2011. Chorismate mutase: an alternatively spliced parasitism gene and a diagnostic marker for three important *Globodera* nematode species. European Journal of Plant Pathology 129: 89-102.