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RAPD analysis of the sexual state and sterile mycelium of the fungus cultivated by the leaf-cutting ant *Acromyrmex hispidus fallax*

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That the symbiotic fungus of leaf-cutting ants only occasionally produces the sexual phase makes their identification confusing. This has occurred so rarely, either in laboratory nests, or in unbalanced field nests, that the possibility of contamination of the fungal garden by other fungi cannot be disregarded. In this paper we describe the formation of several basidiomata in a healthy and free-living nest of the leaf-cutting ant *Acromyrmex hispidus fallax*, the cultivation *in vitro* of the sterile mycelia (isolated from the fungal garden) with their typical inflated tips, and the similarity of both forms confirmed by RAPD analysis of their genomic DNA. The fungus was identified as *Leucoagaricus gongylophorus*.

INTRODUCTION

New World myrmicine attini comprise 200 or so species of fungus-growing ants (Bolton 1995) and, among them, 37 leafcutter species exploit living plants for food. Besides the plant sap they intake from leaves, they obtain a complete and balanced diet from the associated fungi. *Atta* and *Acromyrmex* are considered the most evolved genera among leaf-cutting ants, and their nests can have hundreds of underground chambers, most of them filled with fungus gardens.

Möller (1893) first described the isolation of fungi from various nests of Attini. He also described fruiting-bodies occurring on the surface of some nests, and named them *Rozites gongylophora*. These fungi have never been found living outside ants' nests, probably because they depend on the ants which have developed several mechanisms to protect their mutualistic partner from competitors, including physical and chemical weapons (Hervey & Nair 1979, Hölldobler & Wilson 1990, Iwanami 1978, Schildknecht & Koob 1971). Phylogenetic studies have shown the coevolution of the partners (Chapela *et al*. 1994, Hinkle *et al*. 1994). When cultivated *in vitro* these fungi exhibit a slow growth-rate (Cazin, Wiemer & Howard 1989, Powell & Stradling 1986) and are often easily overrun by contaminants. Worker ants are continuously pruning the mycelium and thus the formation of a perfect state in healthy nests is very difficult to find but the absence of ant care, usually caused by disturbance in the nest by digging, or death of the queen (Weber 1938, 1966) may trigger basidiomata development.

Fungal sporophores collected from ants' nests have been classified into different genera and species. As pointed out by Powell & Stradling (1986) and Fisher, Stradling & Pegler (1994a), one of the problems of fungus gardens is to elucidate whether in the few times when basidiomata are found (usually in declining nests), they are in fact the sexual stage of the ant fungus or some contaminant.

Fruiting-structures were observed *in vitro* by Weber (1957, 1966), but only with some of the primitive attini. Weber reported that the fungus associated with *Cyphomyrmex costatus* was a *Lepiota* and the fungus isolated from an *Apterostygma mayrii* nest was considered to be an *Auricularia*, perhaps *A. polytricha*. Bononi, Autori & Rocha (1981) observed a fruiting-body in a laboratory nest of *Atta sexdens rubropilosa* which was identified as *Leucocoprinus gongylophorus*. Many investigators have adopted the name *Leucoagaricus gongylophorus* proposed by Singer (1986). Indeed, Weber (1977) and Romero, Chacon & Guzmán (1987) have given a good picture of how this matter has been considered over the last few years. According to Mueller, Rehner & Schultz (1998) these fungi belong to the *Lepiotaceae* and the current name given to the perfect form is *Leucoagaricus gongylophorus* (North, Jackson & Howse 1997).

Some researchers have recently described the formation of basidiomata in nests of attini reared in the laboratory.

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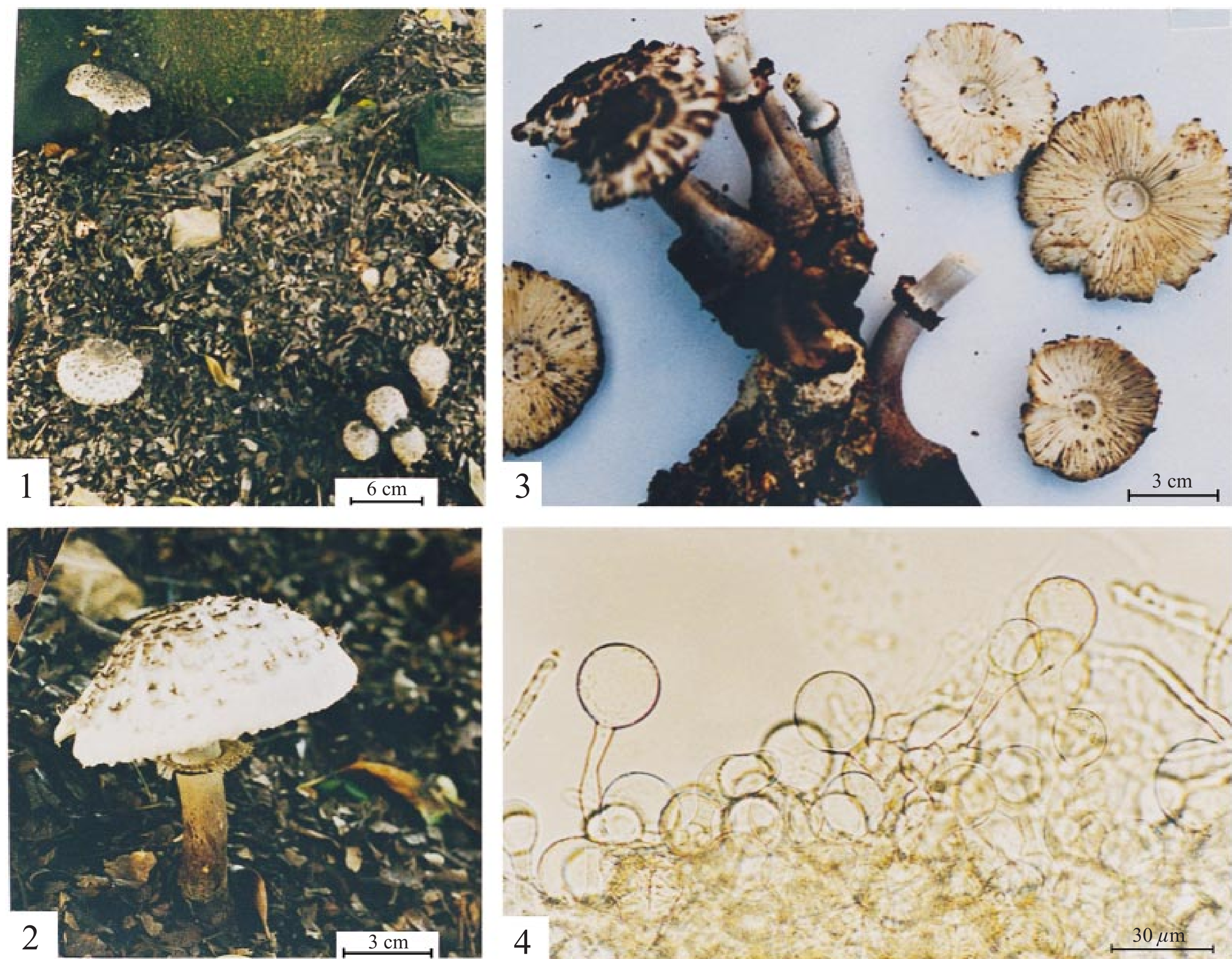

Figs 1–4. *Leucoagaricus gongylophorus*. **Fig. 1.** Partial overview of the site showing several fruiting-bodies, including two already open and a cluster of four still closed. **Fig. 2.** Areolate basidiocarp with brown dots and a ring at the stalk. **Fig. 3.** Mycelia and gregary basidiomata from which they grew; some caps were detached to show the gills. **Fig. 4.** Gongylidia observed on the pure culture of fungus isolated from the nest where the basidiomata had grown.

Muchovej, Della Lucia & Muchovej (1991) reported the formation of basidiomata by the fungus cultivated by the leaf-cutting ant *Atta sexdens rubropilosa*, and Fisher *et al*. (1994a, b) observed a similar occurrence in a laboratory nest of *A. cephalotes*. The latter showed an association between the gongylidia of the vegetative mycelium and basidiomata. In spite of the similarities between such events, these fungi were named *Leucoagaricus weberi* and *Leucoagaricus gongylophorus* respectively, and thus one of the most important problems in elucidating this matter remains unsolved as they continue to be classified on morphological characteristics (North *et al*. 1997).

No previous report has attempted to associate the sterile mycelium and the sexual stage by molecular markers. In 1996, a free-living nest of *Acromyrmex hispidus fallax* produced basidiomata on two occasions (February and April). A small piece of the fungal garden was taken and cultivated in our laboratory giving rise to sterile mycelia. This paper describes the interrelationship between both sexual and mycelial tissue supported by RAPD analysis.

MATERIAL AND METHODS

Ant nest

A healthy nest was found in the backyard of a house in the city of Rio Claro, São Paulo, Brazil. It was in the shade of a peach tree, whose leaves were being cut by some worker ants. The nest was covered by dry leaves and some litter. The ant was *Acromyrmex hispidus fallax*, and specimens are deposited in Coleção Entomológica Adolph Hempel, Instituto Biológico de São Paulo.

Basidiomata

In the last week of February 1996, more than a dozen basidiomata developed almost at the same time. Some of them were solitary and others were gregarious. Almost 2 months later, basidiomata occurred again in the same nest and at least ten well developed basiomata were found. Some were collected for the present study.

Fungal isolation

Pieces of mycelium taken near to the basal body were inoculated in petri dishes in a medium (Pagnocca *et al.* 1990) containing chloramphenicol (20 mg %) to avoid bacterial contamination. When a white mycelium started growing, the material was transferred to test tubes (20 × 25 mm) containing the same medium without the antibiotic and incubated at 25 °C in the dark.

RAPD analysis

Samples of basidiomata and vegetative mycelia developed *in vitro* were taken for RAPD analysis. Total genomic DNA was extracted as described by Raeder & Broda (1986). The 10-mers used as random primers in the RAPD were purchased from Operon Technologies (Alameda, CA). According to the manufacturer's details, the 15 primers used in this work were: OPA4, OPA8, OPA13, OPA5, OPA12, OPA17, OPA20, OPA2, OPA19, OPA18, OXPAX15, OPAX16, OPW4, OPW12, and OPW5.

Amplifications were carried out in a Techne Thermal Cycler as follows: the mixture, made up to 25 µl with autoclaved water, contained 4 µl of DNA solution (about 25 ng), 2.5 µl of primer (0.25 mM), 0.4 µl (2.0 units) of enzyme Taq DNA polymerase (Gibco–BRL) and 8.7 µl of 10 mM $MgCl_2$ (final concentration 3.5 mM). Control reactions were run, omitting genomic DNA, in order to distinguish amplified fungal DNA fragments from artifactual results. The mixture was subjected to the following amplification program: 92 ° for 3 min, followed by 40 cycles of 92 ° for 2 min, 40 ° for 1.5 min, 72 ° for 2 min and ending with 5 min at 72 °. Samples of 22 µl of the RAPD products were analyzed by electrophoresis in 1.4 % agarose gels run with 1 × TBE buffer. Gels were stained with ethidium bromide and photographed under uv light.

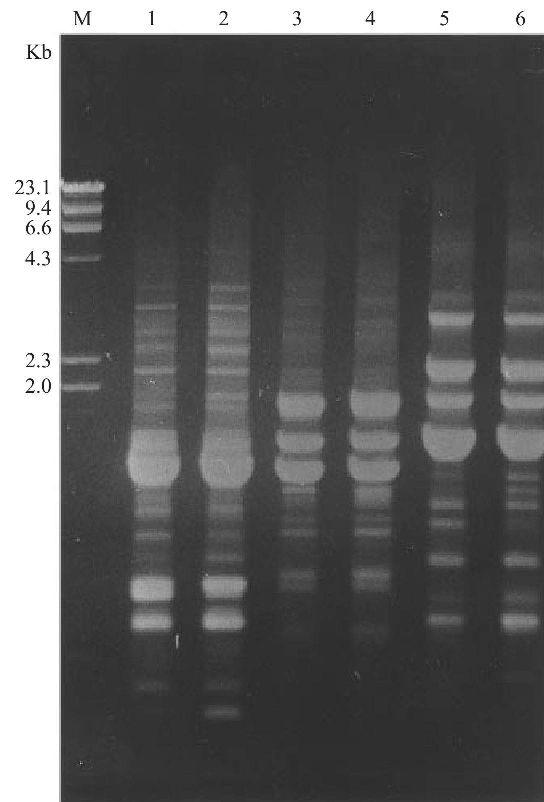

Fig. 5. Random amplification of polymorphic DNA. Lane M, Molecular markers (*Hind*III cut λ DNA); lane 1, basidiomata, primer OPA2; lane 2, vegetative mycelium, primer OPA2; lane 3, basidiomata, primer OPA18; lane 4 vegetative mycelium, primer OPA18; lane 5, basidiomata, primer OPA19; lane 6, vegetative mycelium, primer OPA19.

RESULTS AND DISCUSSION

Fig. 1 shows some basidiomata in different stages of development which were either single (Fig. 2) or gregarious (Fig. 3). The pileus was almost flat and round, and 10–12 cm diam. The hymenium was lamellar with free lamella very close (less than 0.1 cm) to each other. The predominant colour was white with brown dots. The central stipe was 10–15 cm, and was cylindrical and wider (around 3 cm) at the base, smooth, cartilaginous and light brown. Based on morphological characteristics, including the gongylidia observed (Fig. 4) in the white mycelia developed *in vitro*, the fungus was identified as *Leucoagaricus gongylophorus* (Singer 1986).

Random amplified polymorphic DNA (RAPD) techniques (Welsh & McClelland 1990, Williams *et al.* 1990) are being used increasingly to generate molecular markers which are useful in identifying fungi, and differentiate them at the intraspecific level. This molecular technique allows for the detection of a high number of polymorphisms appropriate for analysing relationships of siblings isolated within natural populations. Based on this, RAPD analysis was used to assess the genetic similarity between the basidiomata produced on the fungus garden from the leaf-cutting ant *Acromyrmex hispidus fallax* and the sterile mycelium isolated from the same fungal garden. Since the intensity of amplification products on gel could vary slightly between different DNA samples of the same strain, and even between two identical reactions, one should only consider the absence or presence of intense bands on the gel (van der Vlugt-Bergmans *et al.* 1993). Only those reproducible products, namely 101 loci detected by the 15 primers, were scored as RAPD markers. All of them were shared by the basidiomata collected from the ants' nest and gongylidia-bearing mycelium. Fig. 5 shows the results obtained with the primers OPA2, OPA18 and OPA19. The number of primers utilized and/or the number of polymorphic bands scored, determines the reliability of data collected for genomic similarity studies. According to Colombo *et al.* (1998), the utilization of 10 to 30 primers (50–100 bands) is adequate to estimate genetic relationships within species. Thus, obtaining the same genetic fingerprints from the basidiomata and the sterile mycelia, allowed us to conclude that the sexual form observed in the *Acromyrmex hispidus fallax* nest had developed from the ants' symbiotic fungus.

M. Bacci *et al.* (unpubl.) have worked with our material and with other mycelial forms isolated from four additional species of leaf-cutting ants, and sequenced the coding regions of 18S and 28S ribosomal RNA, as well as the internal transcribed spacer 2 (ITS2). The similarity between the sequences of the

sporophore of the fungus associated with *Acromyrmex hispidus fallax*, and those of the mycelia was 100 % for 18S, 99 % for 28S, and 97–100 % for ITS2 regions; this reinforces our finding that the *L. gongylophorus* sporophore represents the sexual stage of the symbiotic fungus of *Acromyrmex hispidus fallax*.

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