Myrmecomyces annellisae gen. nov., sp. nov. (Deuteromycotina: Hyphomycetes), an endoparasitic fungus of fire ants, Solenopsis spp. (Hymenoptera: Formicidae)

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Two strains of a parasitic fungus, differing only in assimilation of cellobiose and colonial morphology on some media, occur in the haemolymph of fire ants in the United States and Argentina. In the host, obclavate yeast cells bud from a narrow base at the larger pole. A true, septate mycelium without clamp connections readily develops *in vitro*, and has been observed on rare occasions in the host. The hyphal septum has a simple, central pore. Morphology in insect tissue culture medium closely resembles that in the host; however, in simpler media the cells and hyphae are pleomorphic and often only mycelial. Chlamydospores are produced; otherwise, sporulation has not been observed. The morphological, cultural, and physiological characteristics are described, and the fungus is named as a new species, *Myrmecomyces annellisae*, representing a new genus. Gross pathology, histopathology, or changes in host behaviour have not been observed; however, parasitized hosts appear to succumb more readily to stress.

The imported fire ants, *Solenopsis richteri* Forel and *Solenopsis invicta* Buren, were introduced into the United States from South America in *ca* 1920 and 1940, respectively. These medical and agricultural pests now infest over 10^8 ha in 11 southeastern states and Puerto Rico (Lofgren, 1986). Recently, they have been accidently transported to Arizona and California. If they become established in the more humid or irrigated areas of the western U.S.A., their range will increase substantially. In addition, a polygynous form with denser populations and which is sometimes more difficult to control with chemicals is spreading within the population (Glancey *et al.*, 1987).

In the United States, the imported fire ants are essentially free of specific natural enemies (Jouvenaz *et al.*, 1977). In South America, however, they are beset by pathogens, parasites and social parasites, and symbiotic predators (Jouvenaz, 1983). We are studying these organisms in order to select candidates for introduction into the United States. The establishment of a complex of specific natural enemies may effect a permanent amelioration of the fire ant problem. The parasitic fungus described in this paper is one of the few organisms which are symbiotic with the imported species of fire ants in the United States.

MATERIALS AND METHODS

Colonies of *Solenopsis quinquecuspis* Forel and *S. invicta* were collected, respectively, in Buenos Aires Province, Argentina, and Florida, U.S.A. The ants were separated from the soil and examined for pathogens by the procedures described by Jouvenaz *et al.* (1977). Infected colonies of *S. quinquecuspis* were hand-carried (under Florida-USDA permits) to our Gainesville, Florida laboratory. Colonies of both species were maintained soil-free in William's nests (Banks *et al.*, 1981) at *ca* 28 °C in a walk-in rearing chamber. They were fed insectary-reared corn earworm, *Heliothis zea* Boddie (Lepidoptera) larvae, boiled eggs, and honey-agar.

The fungi were isolated from pupae, as these are much easier to dissect than the heavily sclerotized adults. The pupae were surface-sterilized by successive immersions in 95% ethanol (2–3 s), household bleach (3 min), 10% sodium thiosulphate (3–5 min to neutralize free chlorine), and three rinses in sterile, deionized water (Poinar & Thomas, 1978). To submerge the pupae, a small quantity of ethanol was added to the bleach and sodium thiosulphate solutions. The pupae were then placed individually in *ca* 0·3 cm³ of sterile Grace's insect cell culture medium (GCCM; Gibco Labs, Grand Island, N.Y.) and incised (avoiding the gut) to allow haemolymph containing yeast cells to flow into the GCCM. After microscopic confirmation of the presence of yeast cells, a loop of the GCCM was transferred to an insect tissue culture medium (TCM) consisting of 3·5% bovine serum albumen, 10% foetal bovine serum, and 10% egg ultrafiltrate in GCCM (Yunker, Vaughn & Cory, 1967), and also streaked on brain-heart infusion agar (BHI; Difco). Incubation was at 28° (the optimum temperature for fire ants), without agitation. Axenic cultures were stored in TCM in the refrigerator, subcultured monthly, and irregularly passed through *H. zea* larvae by injection.

Two small, healthy laboratory colonies of *S. invicta* (one for
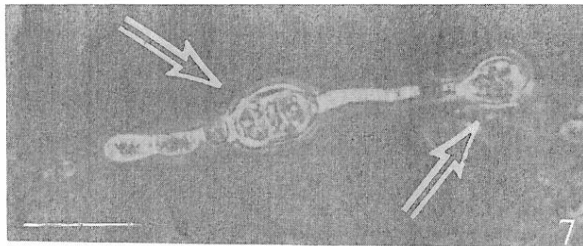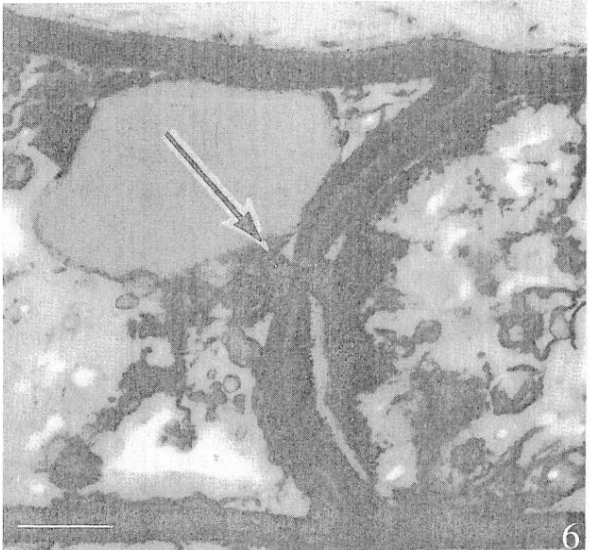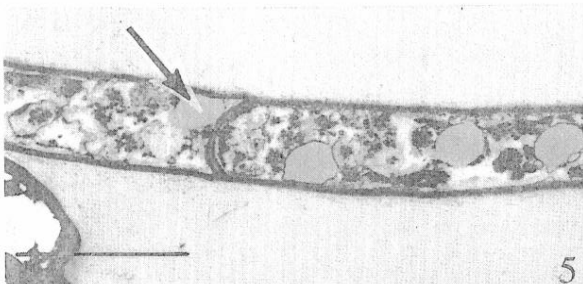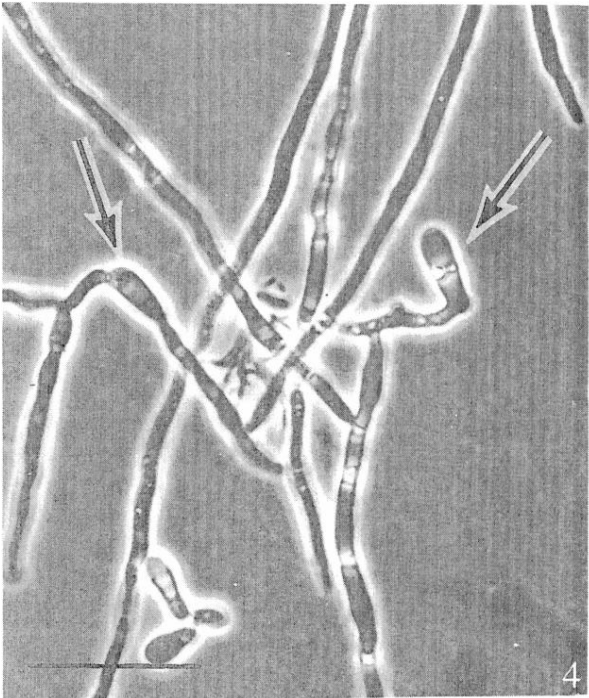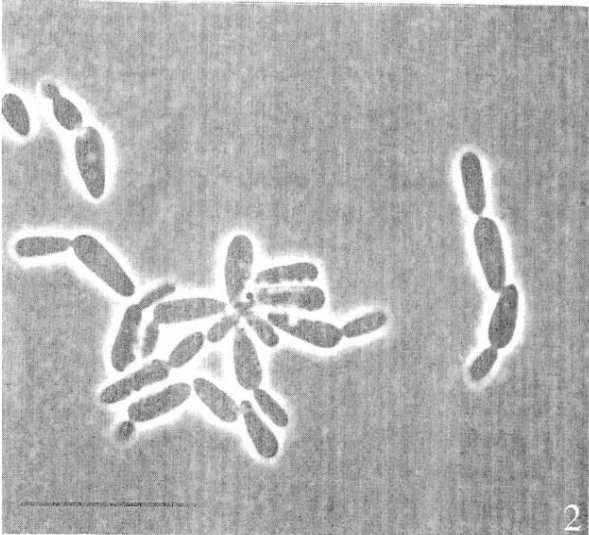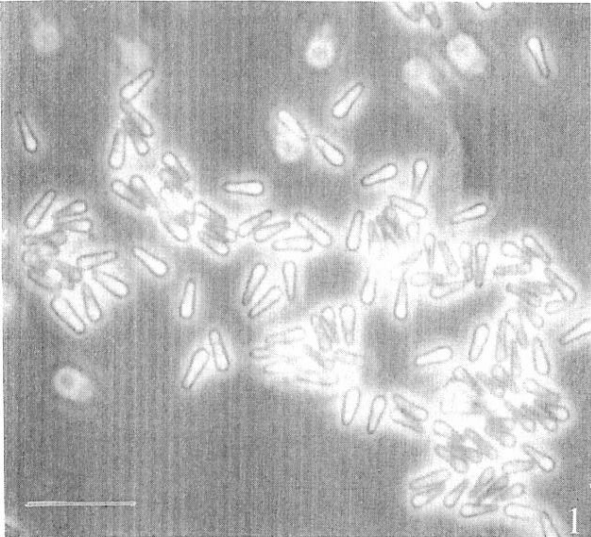
each isolate) were exposed to yeast cells by feeding them a paste of boiled egg yolk and washed, actively growing yeast suspension, and also infected (by injection) *H. zea* larvae, once every two days for four weeks (15 feedings). Ingestion of yeast cells by fourth instar larvae (all other life stages consume only liquids from which even bacteria are removed by filtration; Jouvenaz, unpublished) was confirmed microscopically. Individual and mass-extract wet mounts of pupae were examined for infection by phase-contrast microscopy.

Diagnosis of infection was made by observing yeast cells in fresh smears or in the haemolymph of intact specimens (viewed through the cuticle) held in a drop of water under a cover slip, by phase-contrast microscopy. Measurements were made with a Vickers-A.E.I. image-splitting micrometer. Wet mounts in India ink were used to determine whether cells are encapsulated. Tissue specificity and histopathology were studied by light microscopy of thick sections of specimens prepared for electron microscopy, and also by electron microscopy.

Ant tissue specimens for electron microscopy were prefixed in buffered (0·1 M sodium caeodylate, pH 7·5) 1 % (w/v) osmium tetroxide for 30–60 min at room temperature, rinsed with buffer, and fixed in 2·5 % (v/v) glutaraldehyde-1 % acrolein in 0·1 M sodium cacodylate buffer at pH 7·5. Specimens were washed in buffer and usually stored in Histocon® (Polysciences, Inc., Warrington, PA) in the refrigerator overnight prior to postfixation. Specimens were washed in buffer, postfixed in buffered 1 % (w/v) osmium tetroxide for 2 h at room temperature, washed in deionized water, and stained *en bloc* in 0·5 % (w/v) aqueous uranyl acetate overnight. Specimens were dehydrated with acidified 2,2-dimethoxypropane and infiltrated and embedded in Spurr-Quetol 651 resin.

Yeast cells and mycelia from liquid cultures were fixed for 2·5 h in 2·5 % (v/v) glutaraldehyde in 0·1 M sodium cacodylate buffer, pH 7·5) rinsed in the same buffer, embedded in 2 % agar, and postfixed in 1 % (w/v) aqueous osmium tetroxide. These specimens were dehydrated through an ascending ethanol and acetone series, and embedded in Epon-Araldite.

Thin sections, cut with an LKB ultramicrotome, were post-stained with 2 % (w/v) aqueous uranyl acetate followed by lead citrate. Grids were examined with an Hitachi H-600 electron microscope.

All tests of cultural and physiological characteristics were conducted as described by van der Walt & Yarrow (1984). Morphology was observed in TCM, BHI broth, Pasteur's broth, and glucose-yeast extract-peptone (GYP) water, and on BHI agar, Sabauraud's malt and dextrose agars, Yeast morphology agar, cornmeal agar (all Difco) and Pasteur's agar. The Dalmau technique was employed on cornmeal agar and yeast morphology agar to determine whether pseudomycelia are formed (van der Walt & Yarrow, 1984). In an attempt to

induce sporulation, colonies were incubated at 15 and 25° on reduced concentrations (50, 25, and 12·5 %) of BHI agar, Pasteur's agar, and cornmeal agar.

All physiological tests were conducted in duplicate with each of the two isolates, and in cases of delayed, limited, or ambiguous reactions, were repeated for confirmation. Because these fungi grow slowly, the Diazonium Blue B colour reaction test was conducted on colonies after three weeks incubation (standard procedure) and repeated on different colonies after six weeks incubation. Commerical yeast nitrogen, carbon, and vitamin-free base media (Difco) were used and were sterilized by filtration.

DESCRIPTIVE ACCOUNT AND DISCUSSION

Yeast cells from the two host species could not be distinguished morphologically. Cells from worker haemolymph or TCM were obclavate to ovoid (Fig. 1), and measured 2·9 (2·4–3·3) × 8·7 (7·8–10·4) μm (n = 30 from haemolymph, 60 from TCM for each isolate; width measured at widest point). Yeast cells from worker ants were most consistently obclavate; cells grown in less rich media were correspondingly more pleomorphic and more readily produce mycelia. Yeast cells from the haemolymph of several adult male *S. invicta* were predominantly bacilliform rather than obclavate, and were slightly smaller, measuring 2·3 (2·0–2·8) × 6·5 (5·8–7·1) μm (n = 30). We have not been able to examine yeast cells from male *S. quinquecuspis*.

Yeast cells in haemolymph or TCM reproduced by narrow-based, primarily (> 99 %) unipolar budding from the larger pole (Fig. 2). Budding from the smaller pole was not observed in haemolymph; however, in a sample of 1000 cells in TCM (500 of each isolate, 72 h cultures, 28°), five cells (0·5 %) were budding from the smaller pole and two cells (0·2 %) were budding from both poles simultaneously. In GYP, cells were more pleomorphic, budding from the small pole and bipolar budding were more frequent, and growth of mycelia occurred. In a sample of 1000 cells (500 of each isolate, 96 h cultures, 28°) in GYP, 84 (8·4 %) were budding from the smaller pole and 41 (4·1 %) were budding from both poles simultaneously. Yeast cells *in vitro* tended to cluster in small groups, often adhering by their smaller poles and thereby appearing superficially to bud from these poles; therefore, only isolated, budding cells of unambiguous polarity were examined to determine budding characteristics.

A median section of a cell shows a single nucleus and various organelles (Fig. 3). The yeast cells were not encapsulated.

Branched, septate hyphae grew from either or both poles of yeast cells *in vitro* (Fig. 4). The hyphal septum had a simple, central pore plugged with electron-dense, amorphous material (Figs 5–6); there were no clamp connections. Hyphae grown

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Figs 1–7. *Myrmecomyces annellisae*. Fig. 1. Yeast cells from haemolymph of *S. invicta*. Cells from *S. quinquecuspis* are identical in appearance, bar = 20 μm. Fig. 2. Yeast cells budding in TCM; note narrow base, large pole of mother cell, bar = 20 μm. Fig. 3. Yeast cell ultrastructure; note single nucleus, bar = 1 μm. Fig. 4. Hyphae growing from yeast cells (arrows), bar = 20 μm. Fig. 5. Hyphal ultrastructure; note septum with simple pore, bar = 5 μm. Fig. 6. Detail of septum with simple pore shown in Fig. 5. Note amorphous, electron-dense material plugging pore, bar = 0·5 μm. Fig. 7. Intercalary and terminal chlamydospores from yeast morphology agar, bar = 20 μm.
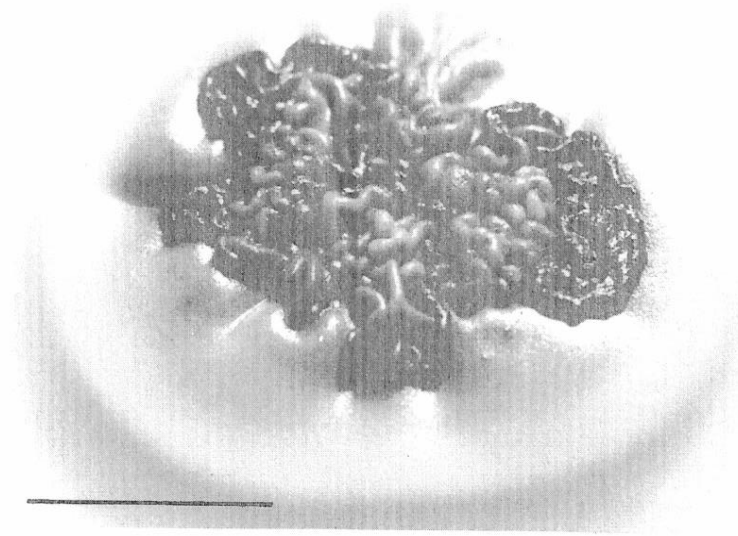
in TCM or BHI broth were *ca* 2·1–2·3 µm diam.; those grown in Pasteur's broth were thicker, *ca* 2·7–3·6 µm. Pseudomycelia did not develop in TCM or BHI broth; however, in GYP water short chains of 6–8 enlarged cells developed from which a true mycelium grew. Round to ovoid intercalary and terminal chlamydospores up to *ca* 22 µm diam. or length developed in hyphae growing in or on the surface of solid media (Fig. 7). Chlamydospores seemed especially abundant on yeast morphology agar.

Colonies on BHI were glabrous. Young colonies were smooth and satiny, ivory white to light beige, with margin entire or very slightly fringed. The texture is firm and slightly flexible. Most colonies developed a convoluted or cerebriform central area which, especially with the isolate from *S. invicta*, may become tarry black (Fig. 8). However, the black pigment may be less intense or diffused, so that the colony may be dirty white mottled with black (occasionally dull, dark green) to uniform grey-black. Colonies six weeks old (from a loop of aqueous suspension, incubated at 28°) were 12–14 mm diam. and were composed of both mycelium and yeast cells. Many of the latter were enlarged, ranging up to *ca* 9 × 22 µm.

Colonies on Pasteur's agar were usually glabrous initially, and sometimes developed a convoluted surface, but none produced black pigment. Instead, fine, sterile hyphae emerged which eventually covered the entire surface of the colony (Figs 9–10). Colonies of the isolate from *S. invicta* tended to be white initially, but usually became tinged with light yellow, whereas those from *S. quinquecuspis* became golden yellow and sometimes developed concentric rings of golden yellow to orange-yellow. The colonies also differed in shape; those from *S. invicta* were usually elevated and rounded, measuring up to *ca* 30 mm diam. and 15 min, high, whereas those from *S. quinquecuspis* measured up to *ca* 20 mm in diam. and 3–4 mm in high, and often developed a central, circular ridge enclosing a depression.

Colonies of both isolates on yeast morphology agar consisted only of mycelium and appeared identical. Initially, they were flat and velvety, with a fringed margin, and were orange-brown. After 5–6 weeks incubation, tufts of light, yellow-tinted hyphae emerged from the surface to eventually cover the colony and become yellow. After 12 weeks, colonies (from a loop of aqueous suspension, incubated at 25°) were 9–10 mm diam. on and below the agar surface, whereas the elevated centre areas (emergent hyphae) were 7–8 mm diam. and 2–3 mm high.

Growth of these fungi *in vitro* was slow (even in TCM), and in some media was absent, sparse, or (compared to *in vivo* or TCM) atypical. Thus, growth did not occur initially in vitamin-free medium; however, in subsequent tests to determine specific vitamin requirements, limited growth of mycelia only occurred in the vitamin-free control and was not enhanced in media containing all or combinations of the following: biotin, calcium pantothenate, folic acid, *m*-inositol, niacin, *p*-aminobenzoic acid, pyridoxine, riboflavin, and

thiamine. In the vitamin tests, the mycelia developed in a few clumps, each of which probably arose from a single yeast cell. Likewise, only or primarily mycelial growth occurred in tests for assimilation of carbon compounds. In the assimilation tests, the mycelium developed as sheets which adhered to the glass; pellicles were not formed. The two isolates differed only in assimilation of cellobiose. Nutritionally rich media (TCM, BHI) favoured multiplication of the yeast phase, which can be maintained free of mycelia indefinitely in these media by periodic subculturing.

Fermentation: negative (no gas; slight acid at four weeks from glucose and sucrose).

Assimilation of Carbon Compounds:

Galactose	−	Raffinose	−	Mannitol	+
Sucrose	+	Sol. Starch	+	Erythritol	−
Maltose	+	D-Xylose	−	Ribitol	−
Cellobiose	*	Arabinose	−	Inositol	−
Trehalose	+	D-Ribose	−	Citric acid	−
Lactose	−	Rhamnose	−	Succinic acid	−

*Isolate from *S. invicta* − ; from *S. quinquecuspis* +
+ = positive reaction
− = no reaction

Splitting of arbutin: +
Assimilation of nitrate: −
Growth in vitamin-free medium: absent or sparse and atypical.
Diazonium Blue B colour reaction: −
Production of urease: − (growth on this medium was poor).
Production of amyloid compounds: no growth on this medium.
Maximum temperature for growth: 32 °C

TAXONOMY

The true yeasts (Endomycetales) that produce mycelia have dolipores (basidiomycetous species) or micropores (ascomycetous species) in the hyphal septa. The known species do not deposit melanin in the cell walls (de Hoog and McGinnis, 1987).

The dematiaceous or black yeasts (Hyphomycetes) produce mycelia with hyphal septa having dolipores (basidiomycetous species) or simple pores which are usually associated with Woronin bodies (ascomycetous species). Melanin is deposited in the cell walls. In addition, many black yeasts are capable of forming a synanamorph consisting of abundant mycelium (de Hoog & McGinnis, 1987).

The dimorphic fungus of fire ants appears to be allied with the ascomycetous black yeasts. The morphology of the septal pore corresponds to that of the ascomycetous black yeasts, and it produces black pigment on at least on one medium. In addition, the fungus of fire ants produces considerable true mycelium, possibly more than is produced by typical mycelial yeats (personal communication, C. P. Kurtzman, USDA, ARS, Peoria, Ill.). The negative Diazonium Blue B colour tests

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Figs 8–10. *Myrmecomyces annellisae*, colony growth. **Fig. 8.** Colony on Difco brain-heart infusion agar. Note convoluted central region; smooth perphery is at or under agar surface. Black pigment in centre does not always develop or may be less intense or diffuse, bar = 5 mm. **Figs 9–10.** Colonies on Pasteur's agar. The younger colony (Fig. 9) is just beginning to develop sterile, emergent hyphae, which resemble fine hairs growing from the convolutions, bars = 1 mm.

supports the ascomycetous nature of the fungus (Van der Walt & Yarrow 1984). We are unable to place this fungus in any established genus; therefore, we assign it to a new genus of unknown relationships having the following characteristics:

Myrmecomyces Jouvenaz & Kimbrough, gen. nov.

Etym.: *myrmos* (Gr.), in reference to ant.

Cellulae fermenti ovoideae usque ad obclavatas; auctae ad polum magnum, geminatione singulari aut duplici, e basi angusta. Mycelium rerum, ramosum, septatum, sine fibulis, crescens in vitro; septae poris centralibus et simplicibus. Pseudomycelium deest. Coloris experimentum Diazonium caeruleum "B". Fermentatio deest. Nitras non assimilatur, arbutinum fissum. Pigmentum nigrum crescens in agaro, infuso cerebro et corde. Pigmenta alba aut aurea usque ad aurantia in agaro Pasteuri aut in agaro morphologiae fermenti.

Sp. typ.: *Myrmecomyces annellisae* Jouvenaz & Kimbrough (holotypus).

Yeast cells ovoid to obclavate; reproduce by unipolar or bipolar budding from a narrow base. True, branched, septate mycelium lacking clamp connections develops *in vitro*; septa with central, simple pore. Pseudomycelium absent. Diazonium Blue B colour test negative. Fermentation negative. Nitrate not assimilated. Arbutin is split. Black pigment usually develops on brain-heart infusion agar, white or yellow to orange pigments on Pasteur's agar or yeast morphology agar.

The isolates from *S. quinquecuspis* (Argentina) and *S. invicta* (Florida) differ only in assimilation of cellobiose (in which growth, if it occurs, is limited to mycelia) and in colonial morphology on some media. Therefore, we regard them as strains of one species having the following characteristics:

Myrmecomyces annellisae Jouvenaz & Kimbrough, sp. nov
(Figs 1–10)

Etym.: *annellisae*, in honour of Ms E. Ann Ellis, a former colleague.

Parasitatur in haemolympha igniformicarum, Solenopsis spp. Cellulae fermenti; de cultura medii texturae hospitis ovoideae usque ad obclavatas; 2·9 (2·3–3·3) × 8·7 (7·8–10·4) μm. Reproductio in cultura medii texturae insecti geminatione singulari ad polum magnum, e basi angusta. Coloniae in agaro, infuso cerebro et corde, habentes paginam centralem convolutam aut cerebriformem, nullis myceliis emergentibus, saepe pigmento nigro. In agaro Pasteuri, myceliis emergentibus sterilibus, albus aut aureis usque ad aurantia. In agaro morphologiae fermenti; mycelia sterilia initio testacea, postea aurea. Chlamydosporae fortasse in pagina et infra paginam medii. Sucrosum, maltosum, trehalosum, mannitol, et amylum solubile assimilata, modo myceliis crescentibus. Cellobiosum assimilatum aut non; modo myceliis crescentibus. Galactosum, lactosum, raffinosum, D-xylosum, arabinosum, D-ribosum, rhamnosum, erythritol, ribitol, inositol, acidum citricum et acidum succinicum non assimilata. In mediis sine vitaminis non crescens aut sparsim et solum mycelia. Temperatura maxima crescendi 32° C.

Holotypus: University of Florida Herbarium FLAS 55591.

Isotypus: National Fungus Collection 1102636. USDA, ARS, BARC, Beltsville, MD., USA 20705

    Cultures: American Type Culture Collection, Rockville, MD, USA

    Northern Regional Research Laboratory, USDA, ARS, Peoria, Ill., USA. NRRL 20627.

Type locality: Roadside, County Road 125 *ca* one mile east of US 301, north of Lawtey, Bradford County, Florida, U.S.A.

Collected: 23 February 1989 by Lloyd R. Davis, Jr.

Host: *Solenopsis invicta* Buren [Insecta: Hymenoptera; Formicidae]. Determined by D. P. Jouvenaz.

Yeast cells in worker ants or insect tissue culture medium are ovoid to obclavate; 2·9 (2·3–3·3) × 8·7 (7·8–10·4) μm; pleomorphic on simpler media. Reproduction *in vivo* and in insect tissue culture media by unipolar budding from a narrow base at the large pole; limited budding from small pole may occur in less nutritious media. Mature colonies on brain-heart infusion agar glabrous, usually with convoluted or cerebriform central surface; often with black pigment. On Pasteur's agar covered with emergent, white or light yellow to golden yellow to orange, sterile mycelia. On yeast morphology agar initially orange-brown, later yellow, sterile mycelia; yeast cells absent. Terminal and intercalary chlamydospores may occur in hyphae on surface and subsurface of agar media. Sucrose, maltose, trehalose, mannitol, and soluble starch assimilated; mycelial growth only. Cellobiose assimilated or not; mycelial growth only. Galactose, lactose, raffinose, D-xylose, arabinose, D-ribose, rhamnose, mannitol, erythritol, ribitol, inositol, citric acid and succinic acid not assimilated. Growth absent or sparse and limited to mycelia in vitamin-free media. Maximum temperature of growth 32°.

Additional material: Univ. Fla Herbarium FLAS 55592

    National Fungus Collection 1102635.

    NRRL 20626

Locality: Roadside, junction of RN 9 and RP 51, Ramallo, Buenos Aires Province, Argentina.

Collected: 22 October 1987 by D. P. Wojcik and D. P. Jouvenaz.

Host: *Solenopsis quinquecuspis* Forel. Determined by D. P. Wojcik; confirmed by chemotaxonomy by R. K. Vander Meer.

PATHOBIOLOGY

Myrmecomyces annellisae occurs in the haemolymph of fire ants, *Solenopsis* spp. In addition to the strains from *S. invicta* and *S. quinquecuspis* reported here, we have observed morphologically indistinguishable yeasts in *S. richteri* in Argentina. Although a true mycelium develops *in vitro*, only the budding yeast form is usually observed *in vivo*. Short segments of hyphae have been observed in wet mount smears of adult ants on rare occasions; hyphae have not been observed emerging from hosts. Infected ants do not exhibit gross pathology, histopathology, or altered behaviour; however, they appear to succumb more readily to stress. Thus, *M. annellisae* appears to be primarily a nutritional burden to its host, and is best described as an endoparasite (Evans, 1988).

It is possible that haemolymph circulation may become mechanically impaired in very heavily infected individuals.

The mode of transmission of infection is unknown. Both colonies fed yeasts became infected, but the intracolonial incidence of infection was low. In one colony, only 2 of 200 pupae examined individually were infected. The concentration of yeast cells in a mass extract of *ca* 500 pupae from each colony indicated a similar incidence of infection. The low rate of transmission may be due to loss of invasive ability during *in vitro* culture (a common occurrence with entomogenous fungi), or conidia may be produced under as yet unknown conditions.

In an early survey, Jouvenaz *et al.* (1977) detected *M. annellisae* in 93 (9·24%) of 1007 colonies of *S. invicta* from the United States (the strain discussed herein is a recent isolate). The local abundance of infected colonies was variable. Interestingly, *M. annellisae* was not detected in 2002 colonies of fire ants (primarily *S. invicta*) from the states of Mato Grosso and Mato Grosso do Sul, Brazil, the presumed aboriginal range of this species. However, we detected *M. annellisae* in 11 (0·9%) of 1205 colonies of *S. quincuecuspis* and *S. richteri* in Argentina (unpublished). We have not observed this fungus in over 500 colonies of the tropical fire ant, *Solenopsis geminata* (Fab.), a Caribbean species either native or introduced several centuries ago into Flordia.

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