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Muscodor yucatanensis, a new endophytic ascomycete from Mexican chakah, *Bursera simaruba*

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Abstract -- During a study on the fungal endophytic associations with some trees of the secondary forest of El Eden Ecological Reserve located in the northeastern Yucatan Peninsula of Mexico, a new fungal species was isolated as an endophyte of a tree named chakah, chachah, or hukúp (*Bursera simaruba*) by indigenous Mayas. This fungus is characterized by producing a strong musty odor and absence of reproductive structures. Cultures of this fungus on PDA form a whitish, flocculose colony with an uncolored reverse and a mycelium that grows slowly. Scanning electron microscopy photographs showed in aerial and submerged mycelium the early formation of unique intercalary swollen, thin-walled, rugulose hyphae. Based on morphological and DNA sequence analyses, the Mexican isolate is a member of the *Xylariales* with high similarity to *Muscodor albus* and the related species *Muscodor vitigenus*, but with distinct differences that is here described and illustrated as *Muscodor yucatanensis* sp. nov.

Key words — angiospermous trees, *Burseraceae*, fungal biodiversity, taxonomy, tropical forests

Introduction

The description of *Muscodor*, an endophytic ascomycete genus, constitutes a valuable contribution to our knowledge of fungal biodiversity (Worapong et al. 2001). *Muscodor* species produce a mixture of volatile organic compounds with strong biological activity against bacteria, fungi, and insects (Daisy et al. 2002a, Grimme et al. 2007, Mercier & Smilanick 2005, Ramin et al. 2007, Stinson et al.

2003, Strobel et al. 2001). The type species, *M. albus* was originally described from branches of *Cinnamomum zeylanicum* from Honduras (Worapong et al. 2001). Shortly thereafter, *M. roseus* was isolated from *Grevillea pteridifolia* and *Erythrophleum chlorostachys* from Northern Territory of Australia (Worapong et al. 2002), *M. vitigenus* was obtained from *Paullinia paullinioides* from the Peruvian Amazon rainforest (Daisy et al. 2002b), and *M. crispans* was recorded from *Ananas ananassoides* from the Bolivian Amazon basin (Mitchell et al. 2008). The exploration of tropical ecosystems to discover new fungi for bioprospecting has resulted in new records of *M. albus* from different plants from Thailand (Sopalun et al. 2003), Northern Territory of Australia (Ezra et al. 2004), Indonesia (Atmosukarto et al. 2005), and Ecuador (Strobel et al. 2007). Because *Muscodor* species do not form reproductive structures and their 5.8S rDNA sequences are highly similar, species in this genus have been described based on detailed analyses of colonies, hyphal morphology, and the chemical structure of volatile compounds (Seifert et al. 1995, Taylor et al. 1999). In this work, a new species *M. yucatanensis* is proposed.

Materials and methods

Study area and sample collection

The Eden Ecological Reserve is located in the State of Quintana Roo in the northeastern part of the Yucatan Peninsula of Mexico, at 21°36'–20°34'N and 87°06'–87°45'W. The tree *Bursera simaruba* (L.) Sarg. (*Burseraceae*) is 35 m tall and its fruit, flower, leaf, and bark are used by indigenous Mayas to treat snakebites, skin mycoses, fever, and diarrhea because of its anti-inflammatory, analgesic, antibacterial, and antifungal capabilities (Gómez-Pompa et al. 2003). Host trees were randomly selected and separated from one another by approximately 50 m. Four asymptomatic, healthy mature leaves (6 mo old) from each of three *Bursera simaruba* individuals were collected and transported to the laboratory in sterile Zip-lock® plastic bags and processed within one hour of collection.

Endophytic fungus isolation, description, and preservation

In the El Eden Ecological Reserve laboratory the collected leaves were washed in running sterile distilled water for 60 sec. Each washed leaf was cut into 2 × 2 mm segments with sterile scissors. Leaf segments were surface-sterilized by sequential washes in 0.525% sodium hypochlorite (2 min) and 70% ethanol (2 min), rinsed with sterile distilled water, and then surface-dried under sterile conditions (Arnold et al. 2001). Eight sterilized segments were plated on MEA (agar 20 g, malt extract 20 g, distilled water 1L) supplemented with 4 g/L streptomycin sulfate and 5 mg/L Cyclosporine A (Dreyfuss 1986). Five Petri dishes were prepared for each leaf, incubated under lab conditions, and checked daily for 4 wks. Fourteen morphologically different isolates were obtained. Among the fungi recovered was isolate B110, which showed strong inhibition and produced a musty odor. This fungus did not produce spores on different test media even with added sterilized bark and leaves from *B. simaruba*. The morphology of this fungus was examined using light microscopy, fluorescent microscopy, and scanning electron

microscopy (Goh & Hanlin 1994). For fluorescent microscopy, fungal cells walls were stained with 0.1% w/v calcofluor (Sigma) (Kuck et al. 1981). For preservation, a living culture of this fungus was stored in liquid nitrogen vapor in cryoprotectant (10% v/v) glycerol in distilled water. Culture was deposited in the Herbario Nacional (MEXU).

DNA sequence analyses

The internal transcribed spacer (ITS1-5.8S rDNA-ITS2) region of nuclear ribosomal DNA from strain B110 was amplified and sequenced using primers ITS5 and ITS4 and analyzed as previously described (Glenn et al. 1996). Sequencing was performed by the United States Department of Agriculture–Agricultural Research Service South Atlantic Area Sequencing Facility (Athens, GA, USA). The DNA sequence was deposited in GenBank (www.ncbi.nlm.nih.gov) as accession FJ917287. ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) was used to generate a DNA sequence alignment between strain B110 and other GenBank accessions. Distance-based analysis of the ITS sequence alignment was performed using MEGA 4.1 with the following settings: Kimura 2-parameter model, neighbor-joining algorithm, pairwise deletion of gaps and missing data, and 1000 bootstrap replications.

Results

Taxonomic description

Muscodor yucatanensis M.C. González, Anaya, Glenn & Hanlin, **anam. sp. nov.**

MycoBank # 513288, GenBank # FJ917287 — Figures 1–11

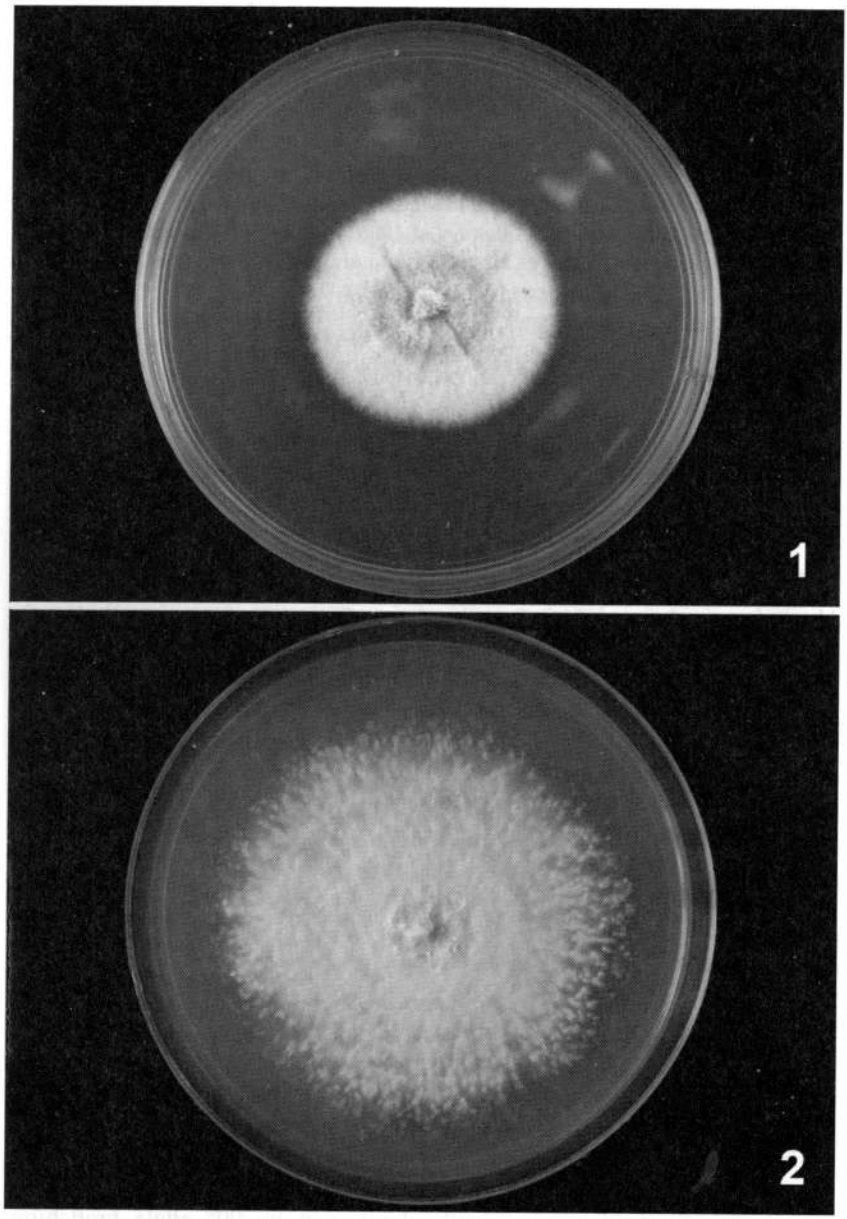
Coloniae in agaro decoto tuberorum (PDA), lente crescentes, ad 30–35 mm diametro attingentes in 14 diebus ad 25°C, albo-flocculosae, odorem mucidum proprie producens. Mycelium sterilibus, ex asexual et sexual sporae vel sporiferus structura ignota. Coloniae vetius (60 diebus), eborinus, aversa parte incolorata, paulo funiculosae. Hyphae hyalinae, leptodermica, rugulosae, septatae, 0.5–4 µm diametro, saepe ramificatione in angulis 90° plerumque, convolventes, fila funiformia 2–20 µm diametro et spiras formantes 10–40 µm diametro, mox vesicula subglobosa intercalaribus numerosa efficientibus.

Teleomorpha ignota. Data sequentia regionis ITS (ITS1-5.8S rDNA-ITS2) Muscodor yucatanensis *(GenBankea accederem # FJ917287) affinitatem* Xylariales *suggerunt.*

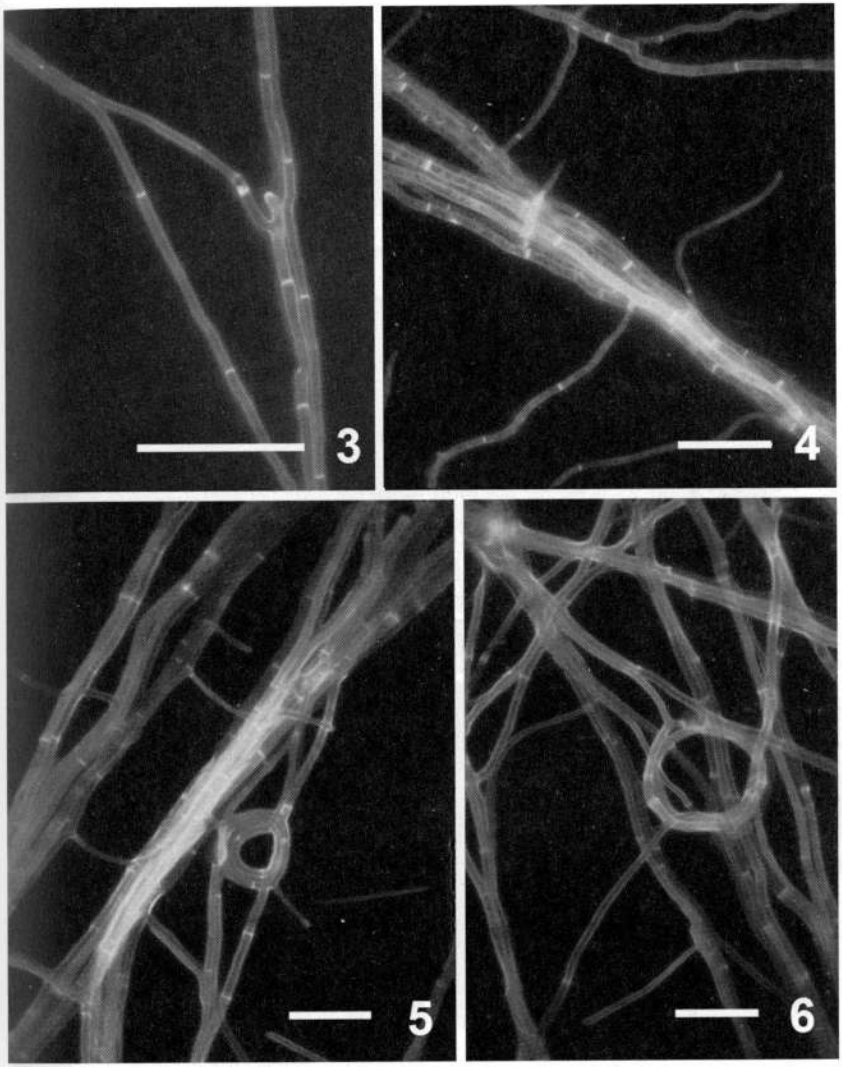
Holotype: MEXICO. Quintana Roo: Isla Mujeres Municipality, El Eden Ecological Reserve (21°13'N 87°11'W), from leaves of *Bursera simaruba*, May 2004, *MC González, AL Anaya*. MEXU 25511.

Etymology: The epithet *yucatanensis* refers to the Peninsula of Yucatan, Mexico.

Colonies on potato dextrose agar (PDA), slowly growing, attaining 30–35 mm diam in 14 d at 25°C, whitish, flocculose and characteristically producing a strong musty odor (Fig. 1). Mycelium sterile, asexual and sexual spores and sporiferous structures unknown. Older colonies (60 days) ivory-white, reverse uncolored, slightly funiculose (Fig. 2). Hyphae hyaline, thin-walled, rugulose, septate, 0.5–4 µm diam, frequently developing by 90° angle branching, intertwining and forming rope-like strands 2–20 µm diam (Figs. 3, 4, 10) and coils 10–40 µm diam, (Figs. 5, 6, 9) soon forming numerous intercalary subglobose vesicles.

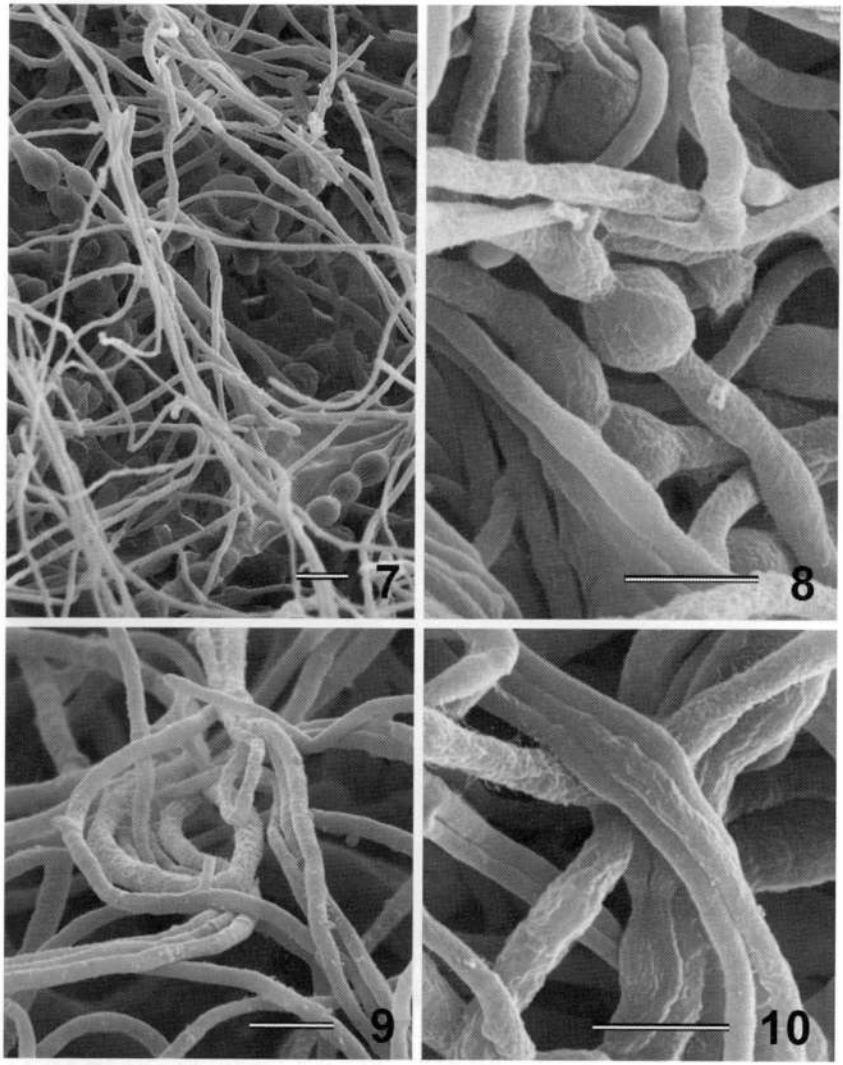


FIGS. 1–2. *Muscodor yucatanensis*. 1. Colony appearance on PDA after 14 days at 25°C. 2. Colony appearance on PDA after 60 days at 25°C.



FIGS. 3–6. *Muscodor yucatanensis*. 3–4. Septate hyphae, showing variation in width, rope-like strands, and intertwining hyphae. 5–6. Rope-like strands and hyphal coil formation. All photomicrographs taken with fluorescent microscopy. Bars = 20 μm.

TELEOMORPH unknown. Sequence data of the ITS regions (ITS1-5.8S rDNA-ITS2) regions of *Muscodor yucatanensis* (GenBank accession # FJ917287) suggest a relationship to *Xylariales*.



FIGS. 7–10. *Muscodor yucatanensis*. 7. Hyphae at surface of colony showing the characteristic swollen cells. 8. Detail of swollen hypha. 9. Hyphal coil formation. 10. A rope-like strand of rugulose hyphae. All photomicrographs taken with scanning electron microscopy. Bars = 5 μm.

FIG. 11. *Muscodor yucatanensis*. Neighbor-joining analysis of aligned ITS rDNA sequences from *Muscodor* species. (Kimura 2-parameter; pairwise deletion). Bootstrap values (1000 replications) are indicated for well-supported clades. All sequences were obtained from GenBank accessions (numbers indicated) except for *M. yucatanensis* strain B110, which we sequenced. Four clades were evident within the *Muscodor* lineage, and these clades appear to correspond to different species. The phylogram is rooted to the *Xylaria* clade, which contains the unidentified endophyte isolates ARIZ B225 and ARIZ B492.

Sequence analyses

Nucleotide-nucleotide BLAST (megablast) query using the 610 bp amplicon sequence against the GenBank nucleotide collection database suggested strain B110 was a member of the *Xylariales* with very high similarity to *Muscodor albus*. Representative ITS accessions of *Muscodor* species and unidentified endophytes were used to determine possible phylogenetic relationships (FIG. 11). Strain B110 clustered in Clade 1 with other unidentified endophytes (99% bootstrap support). This clade is herein recognized as the new species *Muscodor yucatanensis*. Clade 2 includes two unidentified endophytes that also may represent an undefined species (98% bootstrap). Clade 3 includes the previously defined *Muscodor vitigenus* clustering with an unidentified endophyte (99% bootstrap). Lastly, Clade 4 consists of three species, *M. albus*, *M. crispans*, and *M. roseus* (99% bootstrap). Strains ARIZ B225 and ARIZ B492

had identical ITS sequences and represent an unidentified endophyte that here serves as a sister taxon to the delineated *Muscodor* species.

Discussion

Tropical ecosystems probably have a higher biological and functional fungal diversity compared to other climates (Hyde 1997). *Muscodor* is essentially a genus of endophytic, tropical fungi. Its diversity and host range is gradually being revealed as additional hosts and habitats are explored. Species of this genus are found in all tropical regions in Central and South America, South Eastern Asia, and Australia (Worapong et al. 2002, Daisy et al. 2002b, Mitchell et al. 2008, Sopalun et al. 2003, Ezra et al. 2004, Atmosukarto et al. 2005, Strobel et al. 2007). The characteristic mixture of volatile compounds produced by each *Muscodor* species suggests adaptation to a unique ecological role in its respective ecosystem. *Muscodor vitigenus* forms only naphthalene in high concentration, which protects *Paullinia paullinioides* from insects, while different mixtures of volatile compounds produced by *M. albus* or *M. roseus* have a strong and specific activity against a select group of fungi or unique fungal species. For this reason, *M. albus* and *M. vitigenus* were described as different species based on the differences in volatile compounds that they produced. The morphologies of *Muscodor* species show some differences. *Muscodor albus* persistently develops whitish mycelium under light or dark conditions and different media compositions, while *M. crispans* develops pinkish mycelium under light and whitish mycelium under dark conditions; *M. roseus* produces a dense, lightly rose colored mycelium in different media and environmental conditions. Also, hyphal morphology is an additional phenotypical character used to delimit *Muscodor* species. Although *M. albus* and *M. vitigenus* form whitish mycelia, the hyphae of *M. albus* are smaller (1.1–1.7 µm) in diameter than those of *M. vitigenus* (0.7–2.1 µm). *Muscodor crispans* (0.6–2.7 µm diam) forms characteristic undulating hyphae with cauliflower-like structures.

One hundred and six leaf fragments (22%) developed fungal growth and 12 morphologically different isolates were recovered from four leaves of *Bursera simaruba* from the Yucatan Peninsula. Of them, the B110 isolate, which showed strong antifungal activity, was selected for further study. The Mexican isolate on PDA forms a white, flocculose, radially sulcate colony with an entire margin and uncolored reverse with hyaline, rugulose, thin-walled, septate, 90° angle branching hyphae that intertwine and form rope-like strands and coils; it does not form asexual and sexual spores and sporiferous structures. In aerial and submerged mycelium, scanning electron micrographs showed the early formation of unique intercalary swollen, thin-walled hyphal cells. In addition, the fungus from the Yucatan Peninsula produces a distinctive mixture of bioactive volatile compounds and does not form naphthalene (data not shown).

The ITS rDNA sequence data of strain B110 suggested it belongs in *Muscodor* (*Xylariales*) and showed similarity to *M. albus* and *M. vitigenus*.

Although the Mexican fungus isolate B110 characteristics agree with the generic description of *Muscodor*, the possession of unique intercalary swollen thin-walled hyphae, the wider (0.5–4 μm) hyphal diameters, and its rugulose wall, the entire margin of the radially sulcate colonies, the endophytic habit in *B. simaruba* (*Burseraceae*) from medium semideciduous dry tropical forest of the Yucatan Peninsula, the production of new mixture of volatile compounds that does not include naphthalene, and its distinct phylogenetic lineage separate from *M. albus* and *M. vitigenus* all support *Muscodor yucatanensis* as representing an independent new species.

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