

A new endophytic ascomycete from El Eden Ecological Reserve, Quintana Roo, Mexico

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Abstract—During a preliminary survey to report the biodiversity of endophytic fungi associated with leaves of some woody plants from El Eden Ecological Reserve in Mexico, a new fungus was isolated from *Callicarpa acuminata* leaves. Cultures of this fungus on PDA form a white floccose colony with a reddish-brown reverse and a mycelium that develops by 90° angle branches and intertwining of hyphae to form rope-like strands and coils. In addition, this endophytic fungus does not form reproductive structures. Based on morphological and DNA sequence analyses, this fungus is proposed to be a member of the *Pleosporaceae* (*Pleosporales*) and has not been previously described. *Edenia* gen. nov. is described and illustrated to accommodate *E. gomezpompae*.

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

Introduction

Many plants develop endosymbiotic associations with microscopic fungi (endophytes) that colonize symptomlessly the living internal tissues of plants and function as mutualists or commensals (Stone et al. 2000). Other endophytic fungi are latent-infecting fungi; they are antagonistic pathogens capable of causing a minimal level of damage to the host when the latency period ends, thus causing characteristic disease symptoms (Sinclair & Cerkaskas 1997).

Endophytic fungi constitute a resource of undescribed biological diversity and a source of valuable genetic diversity with immediate practical biotechnological applications.

The number of fungal species described worldwide is around 80,060 (Kirk et al. 2001). Of these, only about 7,000 species are recorded from Mexico (Guzmán 1998) even though the country is considered the fourth most megadiverse of the world. Clearly little is known about the biodiversity and ecology of microfungi in Mexico, particularly endophytic fungi from tropical forests. El Eden Ecological Reserve is located in one of the more remote and particularly biodiverse biogeographical zones of Mexico, and the fungal endophytic associations with plant communities in El Eden are unknown. During the past two years a biodiversity project to study endophytic fungi associated with some trees of the secondary forest of El Eden was undertaken including an examination of their secondary bioactive products. In this study, we are describing a new endophytic ascomycete that does not form fruiting structures. Some endosymbiotic fungi do not form spores to aid the species in propagation and dissemination, which makes them difficult to identify morphologically (Bayman et al. 1998). To resolve this taxonomic problem, naming fungi based only on DNA sequence data is accepted by the present code of Botanical Nomenclature (Seifert et al. 1995, Taylor et al. 1999). We describe the new genus *Edenia* gen. nov. to accommodate the new species *Edenia gomezpompa* based on a combination of morphological and DNA sequence data.

Materials and methods

Study area

El Eden was founded as a private ecological reserve in 1990 by botanist Arturo Gómez-Pompa for educational and research activities. The Eden Ecological Reserve consists of 3,700 acres and is located in the State of Quintana Roo in the northeastern part of the Yucatán Peninsula of Mexico, at 21° 36', 20° 34' N and 87° 06', 87° 45' W. The region is covered by several ecosystems: 1) a medium semideciduous dry tropical forest dominated by the chicle tree (*Manilkara zapota*), the chacá (*Bursera simaruba*), tropical cedar (*Cedrela mexicana*), and ramón (*Brosimum alicastrum*), and this forest is the habitat of the spider monkey, jaguar, and many other notable vertebrates 2) low deciduous secondary forest mainly composed of *Leguminosae* and *Polygonaceae* trees, such as *Piscidia piscipula*, *Lysiloma bahamense*, *Acacia cedilloi*, and *Coccoloba* spp. 3) swamp forest 4) savanna 5) microcenotes and cenotes and 6) other wetlands dominated by cattail marsh or sawgrass. The reserve has a mean annual temperature of 24°C, an altitude of 5-10 m, and receives 140-2000 mm of precipitation annually (Gómez-Pompa et al. 2003).

Isolation, description and preservation

Asymptomatic, healthy leaves were collected from *Callicarpa acuminata* Humb. et al. (*Verbenaceae*) from the semideciduous dry tropical forest. This species was selected based on a previous chemical study by Anaya et al. (2003) in which they obtained 5 compounds with moderate biological activity against some phytopathogenic fungi and test plants. A strong surface sterilization protocol was applied to *C. acuminata* leaves (Rodríguez 1994). The complete, intact leaves were immersed in ethanol 75% (1 min), sodium hypochlorite 3.4% available chlorine (10 min), and ethanol 75% (1 min), and then were rinsed with sterilized distilled water and allowed to dry using a sterile absorbent paper. Each surface disinfected leaf was placed separately in a sterile Petri dish sealed with Parafilm and the dishes were transported to the Institute of Ecology at 5 °C and processed after 24 h.

The lamina was cut with the aid of a flame-sterilized scalpel and cork borer into 5 mm diam pieces, and then five discs were plated on potato dextrose agar (PDA: scrubbed and diced potatoes 200 g, dextrose 15 g, agar 20 g Difco, distilled water 1 L) (Hanlin & Ulloa 1988) supplemented with 4g/L streptomycin sulfate, and 5 mg/L Cyclosporine A (Dreyfuss 1986). The Petri dishes were incubated at 25 °C under 24 h dark conditions. Each different isolate was transferred into a culture tube with PDA for further studies. Among the fungi recovered was an interesting isolate named C1c. This fungus was inoculated onto Petri dishes (9 cm diam) with PDA, V8 agar, corn meal agar (Difco), and water agar (agar 20 g Difco, distilled water 1L). We attempted to induce formation of reproductive structures by placing on the agar small pieces of sterilized bark and leaves from *C. acuminata*. Colony colors referred to with the letter M are from Kornerup & Wanscher (1978) color standard.

The morphology of this fungus was examined using light microscopy, fluorescent microscopy, and scanning electron microscopy (Goh & Hanlin 1994). Photomicrographs were taken with an Olympus BX60, Nikon Epifluorescence Eclipse E600 and Jeol JSM 5410-LV microscopes, respectively. For the fluorescent microscopy, fungal cell walls were stained with 0.1% w/v calcofluor (Sigma) according to Kuck et al. (1981). For preservation, a living culture of this new endophytic ascomycete was stored in liquid nitrogen vapor in cryoprotectant (10% (v/v) glycerol in distilled water), at -80 °C in 15% glycerol and at 4 °C in sterile distilled water. Dried cultures have been deposited in the J. H. Miller Mycological Herbarium (GAM) of the University of Georgia and in the fungal collection of Herbario Nacional de México (MEXU) of the UNAM under the accession numbers GAM 16175 and MEXU 25346, respectively.

Molecular procedures

Nuclear ribosomal DNA internal transcribed spacer (ITS) regions (ITS1-5.8S rDNA-ITS2) of strain C1c were amplified and sequenced using primers ITS5 and ITS4 and analyzed as previously described (Glenn et al. 1996). Nucleotide-nucleotide BLAST (megablast) search of the 555 bp amplicon sequence against the nr database of NCBI (www.ncbi.nlm.nih.gov) suggested strain C1c was a member of the *Pleosporaceae* (*Pleosporales*). This was confirmed by performing BLAST searches using just the ITS1 (149 bp) and ITS2 (155 bp) sequences individually to avoid confounding results from the highly conserved 5.8S rDNA sequence.

Taxonomic description

Edenia M.C. González, Anaya, Glenn, Saucedo & Hanlin, gen. nov.
MYCOBANK #510872

Coloniae in agar decocto tuberorum (PDA), celeriter crescentes, primo albae, deinde pallide roseae, reversum rubro-brunnea vel brunneum, velutinae vel floccosae. *Mycelium* sterilibus, ex asexual et sexual spora vel sporiferous structura ignota. *Hyphae* hyalinae, leptodermica, laeves, septatae, saepe ramificatione in angulis 90° plerumque, flexuosae, convolventes, fila funiformia et spiras formantes.

Etymology: Eden + L. suf. -ia, referring to the name of the ecological reserve where the fungus was found.

Colonies on PDA, fast growing, at first whitish, later becoming pinkish white, reverse reddish-brown to brown, velvety to floccose. **Mycelium** sterile, asexual and sexual spores and sporiferous structures unknown. **Hyphae** hyaline, thin-walled, smooth, septate, frequently developing by 90° angle branching, intertwining and forming rope-like strands and coils.

Teleomorph unknown. Sequence data suggest a relationship to *Pleosporaceae* (*Pleosporales*).

Edenia gomezpompae M.C. González, Anaya, Glenn, Saucedo & Hanlin, anam. sp. nov.

MYCOBANK #510944, GENBANK #EF565744

Figs. 1-10

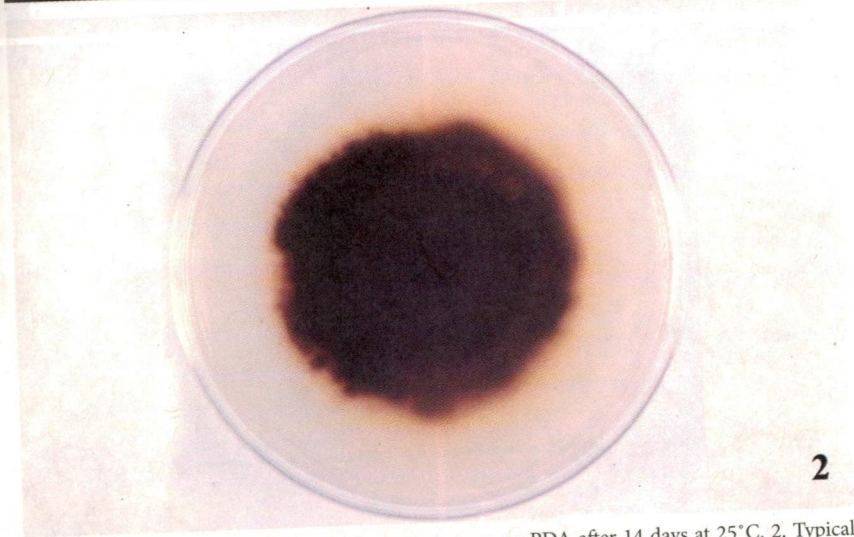
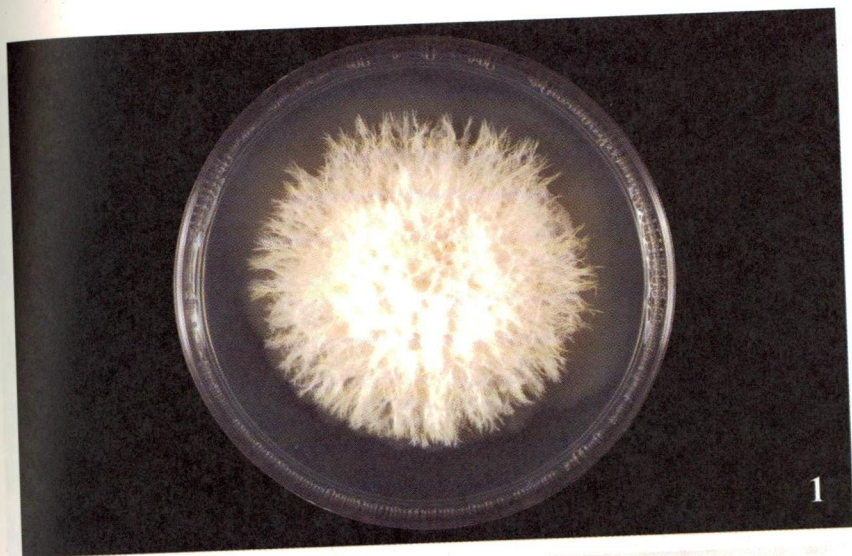
Coloniae in agar decocto tuberorum (PDA), celeriter crescentes 30-35 mm diametro in 7 diebus 25°C, primo albae, deinde pallide roseae [M 7A2], reversum rubro-brunnea vel brunneum [M 9F], velutinae vel floccosae. *Mycelium* sterilibus, ex asexual et sexual spora vel sporiferous structura ignota. *Hyphae* hyalinae, leptodermica, laeves vel undulatae, septatae, 0.6-2.5 µm diametro, saepe ramificatione in angulis 90° plerumque, flexuosae, convolventes, fila funiformia 4-11 µm diametro et spiras formantes 20 µm diametro.

Teleomorpha ignota. Data sequentia regionis ITS (ITS1-5.8S rDNA-ITS2) *Edenia* (GenBank accession # EF565744) affinitatem *Pleosporaceae* suggerunt.

Etymology: The epithet *gomezpompae* refers to the last name of Arturo Gómez-Pompa, an eminent plant ecologist, who has contributed notably to conservation and management of the biodiversity of Mexican tropical forests.

Colonies on PDA, fast growing, attaining 30-35 mm diam in 7 d at 25 °C, at first whitish, later becoming pinkish white [M 7A2], reverse reddish-brown to brown [M 9F8], velvety to floccose. **Mycelium** sterile, asexual and sexual spores and sporiferous structures unknown. **Hyphae** hyaline, thin-walled, smooth to undulate, septate, 0.6-2.5 µm diam, frequently developing by 90° angle branching, intertwining and forming rope-like strands 4-11 µm diam and coils 20 µm diam.

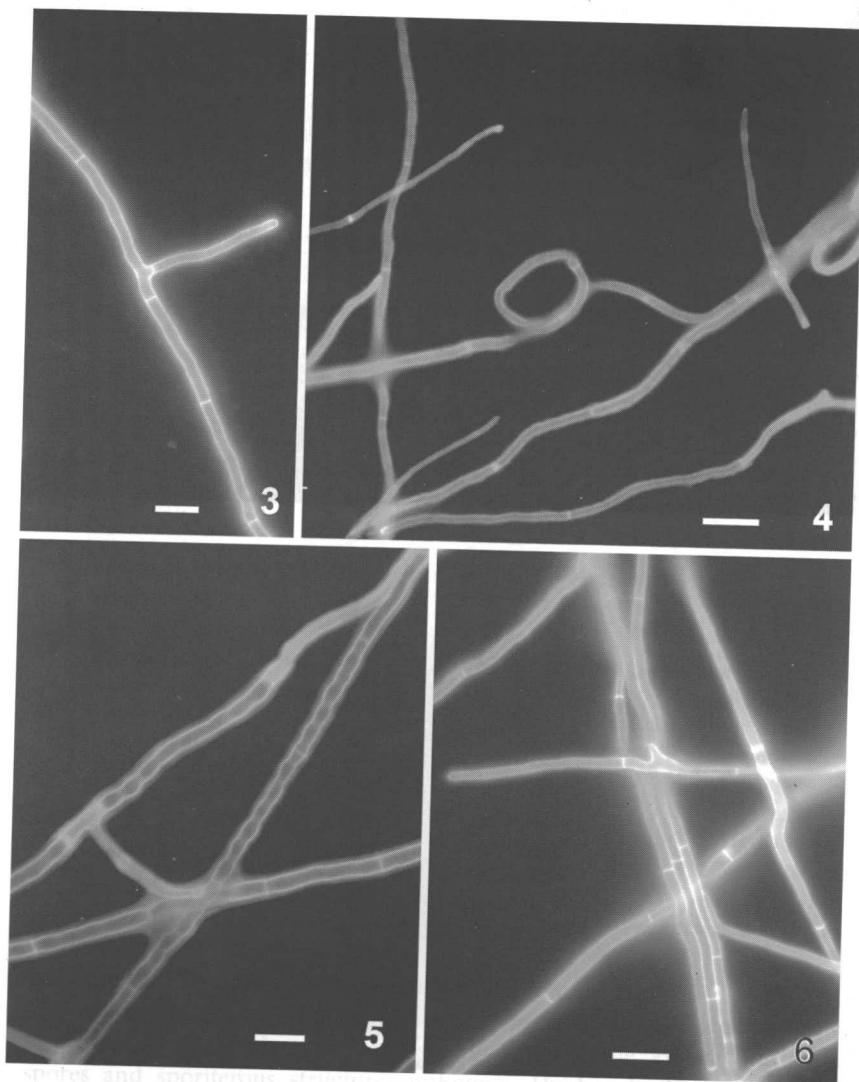
Teleomorph unknown. Sequence data of the ITS regions (ITS1-5.8S rDNA-ITS2) regions of *Edenia* (GenBank accession # EF565744) suggest a relationship to *Pleosporaceae*.



Figs. 1, 2. *Edenia gomezpompae*. 1. Colony appearance on PDA after 14 days at 25°C. 2. Typical reddish-brown pigmentation of culture reverse.

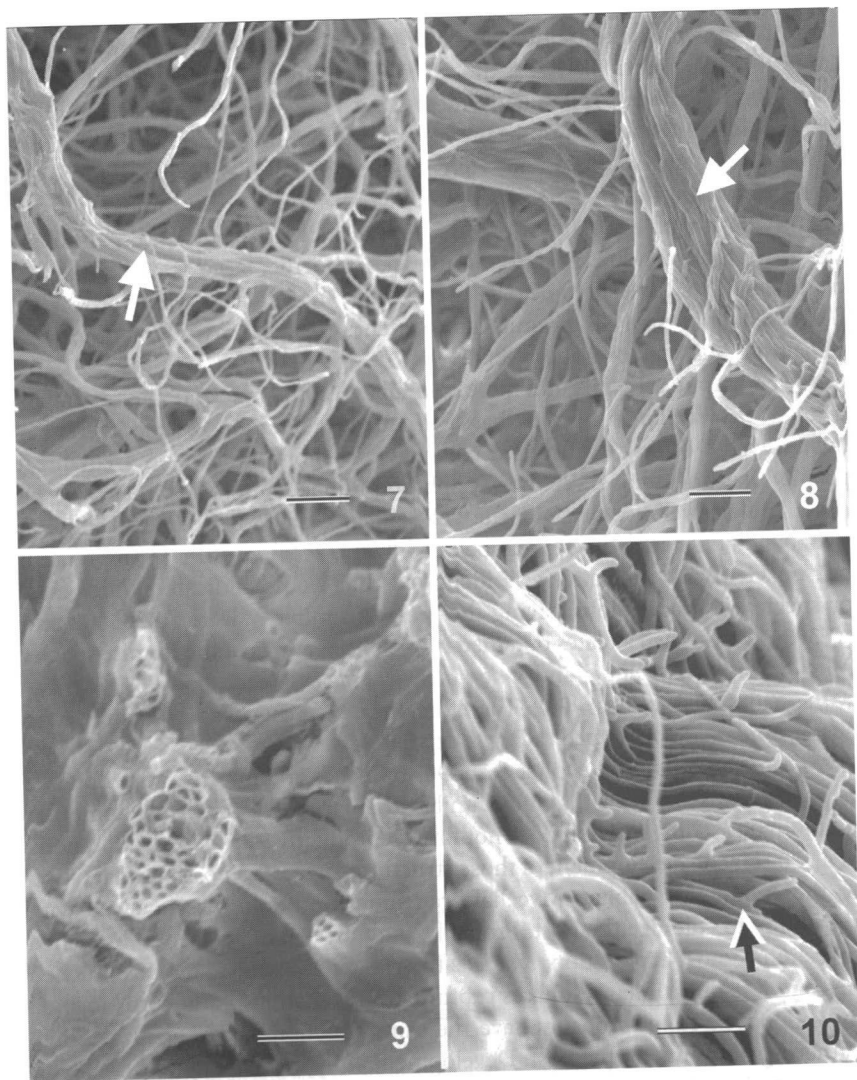
Habitat. Anamorphic ascomycete endophytic within living leaves of *Callicarpa acuminata*.

SPECIMENS EXAMINED — MEXICO. QUINTANA ROO: Isla Mujeres Municipality, EL EDEN ECOLOGICAL RESERVE (87°11'W 21°13'N.), from leaves of *Callicarpa acuminata*, May 2002, A Saucedo-García, AL Anaya. (HOLOTYPE MEXU 25346, ISOTYPE GAM 16175).



Figs. 3-6. *Edenia gomezpompae*. 3. Smooth, septate hypha, with characteristic 90° branching developing. 4. Hyphae showing variation in width and coil formation. 5. Undulate hyphae and 90° branch. 6. Rope-like strands and intertwining hyphae.

Bars 3-6 = 10 μ m. All photomicrographs taken with fluorescent microscopy.



Figs. 7-10. *Edenia gomezpompae*. 7-8. A rope-like strand that exhibits strong cohesion and intertwining of hyphae (arrows). 9. Transverse section of rope-like hyphal structures immersed in agar medium, showing lumina and hyphal-walls. 10. Surface colony showing the characteristic 90° branches vertically oriented (arrow). All photomicrographs taken with scanning electron microscopy.

Bars 7-8 = 20 μ m, 9-10 = 10 μ m.

Discussion

Little is known of the biodiversity and ecology of endophytic fungi in the tropical forests, particularly for those fungi that do not produce reproductive structures in culture. Traditionally, these species are not identified even though they could be new taxa, important ecosystem members, and/or new sources of biotechnological applications. Classical methods of identifying fungi based on morphological characters of reproductive structures are of limited value in identifying fungal endophytes that do not produce these structures, although this is the key to conducting and communicating further basic or applied studies of fungi. At the present time, the use of solid molecular methods involving DNA sequence analysis permits us to identify fungi that might otherwise remain unidentified or undescribed.

Based on phenotypical and molecular analyses, the new endophytic fungus from *C. acuminata* in Mexico has a unique combination of characteristics, including the colony texture and color, hyphal morphology, and a chemical profile that includes three new naphthoquinone spiroketals that will be described elsewhere. Analysis of the ITS sequence data (GenBank accession # EF565744) indicated the fungus belongs to the *Pleosporaceae* (*Pleosporales*), but no GenBank accession was identified that would indicate either genus or species. The accessions having the greatest similarity were AY303602 and AY303611 from two isolates obtained from soil sheetings of the termite species *Macrotermes subhyalinus* and *Odontotermes nilensis* (each had 98% identity with strain C1c; E values = 0.0). Therefore, the new genus *Edenia* is proposed to accommodate the new species *E. gomezpompae*.

Edenia gomezpompae hyphae form 90° branches and intertwining rope-like hyphal strands and coils that look similar to those of *Muscodor albus*, another endophytic ascomycete that does not form reproductive structures (Worapong et al. 2001). The development of those structures suggests a possible adaptation to the endophytic habitat. Mycelial branching morphology is involved with the ability to colonize the host. Probably, the function of sparse 90° branched mycelium is host exploration, whereas the greater abundance of 45° branched mycelium is nutritional resources capture. Mycelial strands and cords formed by endophytic fungi probably have the same function as mycorrhizal fungi, gathering nutrients for the host. Development of hyphal coils can be related to the formation of resistant structures such sclerotia, early stages of development of fruiting bodies and other sporulating fungal structures (Moore et al. 2005, Müller & Krauss 2005). Observations of *E. gomezpompae* and *M. albus*, in planta are needed to compare their hyphal morphology.

The ecological functions of fungi as endophytes, pathogens, and saprobes in the tropics compared to other climates are likely to be similar in some regards, however, tropical ecosystems probably have a higher biological and functional

diversity (Hyde 1997, Van Bael et al. 2005). For example, a particularly interesting fungal association in tropical forests is the fungus that lives in association with termites (Kendrick 1992). The fungus-infected termites build soil biogenic structures named soil runways (soil sheeting) made of soil particles cemented with salivary secretions covering the food source composed of dead plant pieces collected on the soil surface (Bagine 1984). *Macrotermes subhyalinus* and *Odontotermes nilensis* (Macrotermitinae, Termitidae, Isoptera), two main species of fungus-growing termites from semi-arid savanna of Senegal, East African, build soil sheeting structures. When these litter-feeding termites use *Acacia holosericea* leaf litter, the inorganic nitrogen available to plants increased significantly (Deouda et al. 2004).

The ecosystem where *E. gomezpompae* was isolated includes the litter-feeding termite *Nasutitermes mexicanus* (Nasutitermitinae, Termitidae, Isoptera) and *Acacia cedilloi*, an ant-acacia described from the State of Quintana Roo, Mexico (Rico-Arce 1994). The implications of these ecosystem similarities are unknown; however, it does suggest a link between the mycobiota of termite sheetings and leaf fungal endophytes. Much remains to be discovered regarding the fungal community structure and dynamics of the endophytic fungi in the El Eden Ecological Reserve of Mexico.

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