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### Guanophilic fungi in three caves of southwestern Puerto Rico

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#### Abstract:

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Fifty species of guanophilic (bat guano-loving) fungi were isolated from field-collected samples within three caves in southwestern Puerto Rico; most were mitosporic fungi (23 species). The caves studied were Cueva La Tuna (Cabo Rojo), Cueva de Malano (Sistema de Los Chorros, San Germán), and Cueva Viento (El Convento Cave-Spring System, Guayanilla-Peñuelas). The most conspicuous fungus by far was the zygomycete *Circinella umbellata* (Mucorales). *Circinella umbellata* dominated the bat guano incubation chambers (Petri dishes lined with sterile filter paper moistened with sterile water) at ambient laboratory conditions. Nineteen species of basidiomycetes (e.g., *Ganoderma* cf. *resinaceum*, *Geastrum* cf. *minimum*, *Lepiota* sp., *Polyporus* sp., *Ramaria* sp.) and three species of ascomycetes (*Hypoxylon* sp., *Xylaria anisopleura*, and *X. kegeliana*) were also recorded. They were found on soil, rotting leaves, bark and rotting wood, buried in bat guano located below natural skylights or sinkholes.

**Keywords:** coprophilous fungi, bat guano, biospeleology, West Indies, Caribbean

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#### INTRODUCTION

Bat guano or bat guano-enriched soil is one of the most important substrates for fungi in the cave environment along with dung, plant debris, carcasses and other organic debris. Poulson (1972) reported that bat guano habitat is simple in structure but with just enough species to constitute a complete ecosystem. The fungi present commonly serve as saprotrophs and/or pathogens or as transient chemoheterotrophic microorganisms (Northup et al., 1997). It is the fungal biodiversity, its medical or biodegradation potential, and its role as part of the trophic chain in cave ecosystems that attract the attention of mycologists.

During the Spanish governance of Puerto Rico no major efforts were made to study cave fungi, although enormous efforts were devoted to guano mining. In the late 19<sup>th</sup> century, British and American

expeditions led to the exploration of "Dark Cave" or Cueva de Aguas Buenas (Aguas Buenas). Dinwiddie (1899) reported what we suspect should be the earliest mention of fungi in a Puerto Rican cave: "We are all visibly disappointed in that the hanging stalactites are covered with dirt or vegetal fungi of dark brown, which makes the first gallery a dungeon, even with the flaring lights."

Early mycological studies in Puerto Rican caves were focused on the distribution and ecology of the histoplasmosis fungus –*Histoplasma capsulatum*– which is found in soil and bat-roost caves. The first cases of human histoplasmosis in caves of Puerto Rico were diagnosed in 1960 and 1962. Torres-Blasini & Carrasco-Canales (1966a, b) reported *H. capsulatum* from Cueva de los Panes (Utua) and Cueva de Aguas Buenas. This medically important mycosis and its etiologic agent have been documented from Cueva de Aguas Buenas for nearly two decades (Torres-Blasini et al., 1960, 1966a, b; Beck et al., 1976; Carvajal-Zamora, 1977a-c). The dermatophyte *Microsporium gypseum* has been isolated from similar habitats (Tamsitt & Valdivieso, 1970). In addition, the works of Tamsitt & Valdivieso (1970), Stevenson (1975), Magnaval et al. (1984), Nishida (1989), Lewis (1989), Minter et al. (2001), and Ulloa et al. (2006) summarized, commented on or listed the previous mycological reports in caves of the island and the Caribbean.

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Díaz-Martínez (1972) studied eight cases of pulmonary histoplasmosis that were reported in young men who together explored Cueva del Peñón del Barrio Rosario, San Germán, southwestern Puerto Rico. Apparently, this is one of the largest epidemics traced to a single site reported from Puerto Rico. Although Díaz-Martínez (1972 and personal communication, 2007) sent soil samples to Luis A. Roure of the Department of Biology, University of Puerto Rico in Mayagüez for a mycological survey, *H. capsulatum* was not isolated. Díaz-Martínez based his study on cutaneous tests, serological tests, and X-rays.

Nieves-Rivera & Betancourt-López (1994) isolated the water molds *Saprolegnia ferax* and *Achlya americana* from a stream pond known as Charco Azul in El Convento Cave-Spring System of south-western Puerto Rico. Lugo et al. (2001) summarized what is known about the karst of Puerto Rico, including the north and south karstic belts, especially their importance, anthropogenic impact, ecology and geology. However, this important contribution did not comment on any mycological surveys previously carried out in caves of the island.

Nieves-Rivera (2003) conducted a basic mycological survey in the Río Camuy Caves Park and the Río Camuy Cave System in the northern karst of Puerto Rico. He found thirty-nine fungal species and eleven cellular and plasmodial slime molds. Two of the cellular slime molds (*Dictyostelium citrinum* and *D. macrocephalum*) were new records for Puerto Rico.

Landolt et al. (2006) updated the information on dictyostelid cellular slime molds found in caves in nine states of the United States, San Salvador (Bahamas), and Puerto Rico, during 1990 to 2005. Samples of soil material from more than 100 caves were considered. At least 17 species were recovered, although a number of isolates could not be identified conclusively. Four cosmopolitan species (*Dictyostelium sphaerocephalum*, *D. mucoroides*, *D. giganteum*, and *Polysphondylium violaceum*), and a species (*D. rosarium*) with a more restricted distribution were each recorded from more than 25 different caves. Three other species were present in more than 20 caves. The data generated in this study were supplemented with all known published and unpublished records of dictyostelids from caves in an effort to summarize what is known about their occurrence in this habitat.

More recently, Santos-Flores & Nieves-Rivera (2008) and Nieves-Rivera et al. (2008) conducted a preliminary survey of aquatic fungi and related groups in El Convento Cave-Spring System and terrestrial guanophilic fungi in caves of southwestern Puerto Rico (the basis of the present study), respectively. Richardson (2008) isolated 54 species of coprophilous fungi from incubation, in damp chambers, of 21 samples of dung from mammalian herbivores from Puerto Rico and the Lesser Antilles (St. John [USVI], Guadeloupe [France], Dominica and St. Lucia). Although he did not sample caves, many of the species he reported are new records for the region and might be obtained in future samplings. Richardson also discussed the distribution and occurrence of unusual or interesting species.

General data, distribution, evolution, and other aspects regarding Puerto Rican speleobiota related to guano ecology or the production of guano *per se* have been treated by various authors. Refer to the works of Peck (1974, 1981, 1994) for invertebrates and Gannon et al. (2005) and Rodríguez-Durán (2005) for bats. The works of Gile & Carrero (1918), Wadsworth (1977), Cardona Bonet (1985), and Frank (1998) covered aspects of guano production and its repercussions in Puerto Rico and offshore islands. The purpose of this paper is to present the results of our survey and observations on guanophilic fungi and guano-associated fungi in three caves of southwestern Puerto Rico.

## MATERIALS AND METHODS

### Study Sites

Several authors have addressed the Puerto Rican karst surface and its hydrology during past decades (e.g., Monroe, 1976, 1980; Giusti, 1978; Lugo et al., 2001). The southern karst of Puerto Rico differs from that of the north in often being developed on much older limestone rocks (Cretaceous versus Tertiary age), its speleogenesis, and its drier climate (Miller, 2004; Figure 1). The climate of the southern part of Puerto Rico is generally that of a subtropical dry forest (dry forest-volcanic/sedimentary/limestone), but the areas studied fall within the subtropical moist forest (moist-volcanic/sedimentary) according to the Holdridge model (Ewel & Whitmore, 1973; Helmer et al., 2002). The caves studied are described below (see Figure 1).

Cueva La Tuna (also known as Cueva de la Veintidós, Cueva del Maestro Aniceto or Cueva El Maestro, Figure 2) is a hydrologically inactive cave, located in the resistant Late Cretaceous Cotuí Limestone in the Cabo Rojo municipality. It is near the top (about 200 m asl) of a hill in the Monte Grande area, approximately 3.5 km SE of Cabo Rojo, and is within a karst area of caves and a major doline. This cave has about a half kilometer of large passage (8-20 m wide), generally steeply-inclined (total relief about 50 m). The cave has primarily developed down a NE structural dip, but with a significant section along the strike. Much of the floor is dominated by ceiling collapse. Large wall scallops show very slow groundwater dissolved the cave, in hydrologic conditions very different from today. Currently, only infiltrating rainfall enters the cave. Like most large caves in Puerto Rico, it appears to show more than one hydrologic base level in the past. Peck (1981) described the cave as having a chamber with a temperature of 25.5°C at the base of the entrance slope. This ascends to a high ceiling bat chamber. An *Artibeus jamaicensis* bat nursery with temperatures of 29°C and higher is in a side passage near the cave front. Peck (1981) also documented the invertebrate fauna of this cave.

Cueva de Malano (Figure 3) (also known as Cueva de Los Santos or Cueva de Los Braceros) is another hydrologically inactive cave, part of Sistema de Los Chorros of the San Germán municipality (Miller, 2004). This cave system is one of the largest in

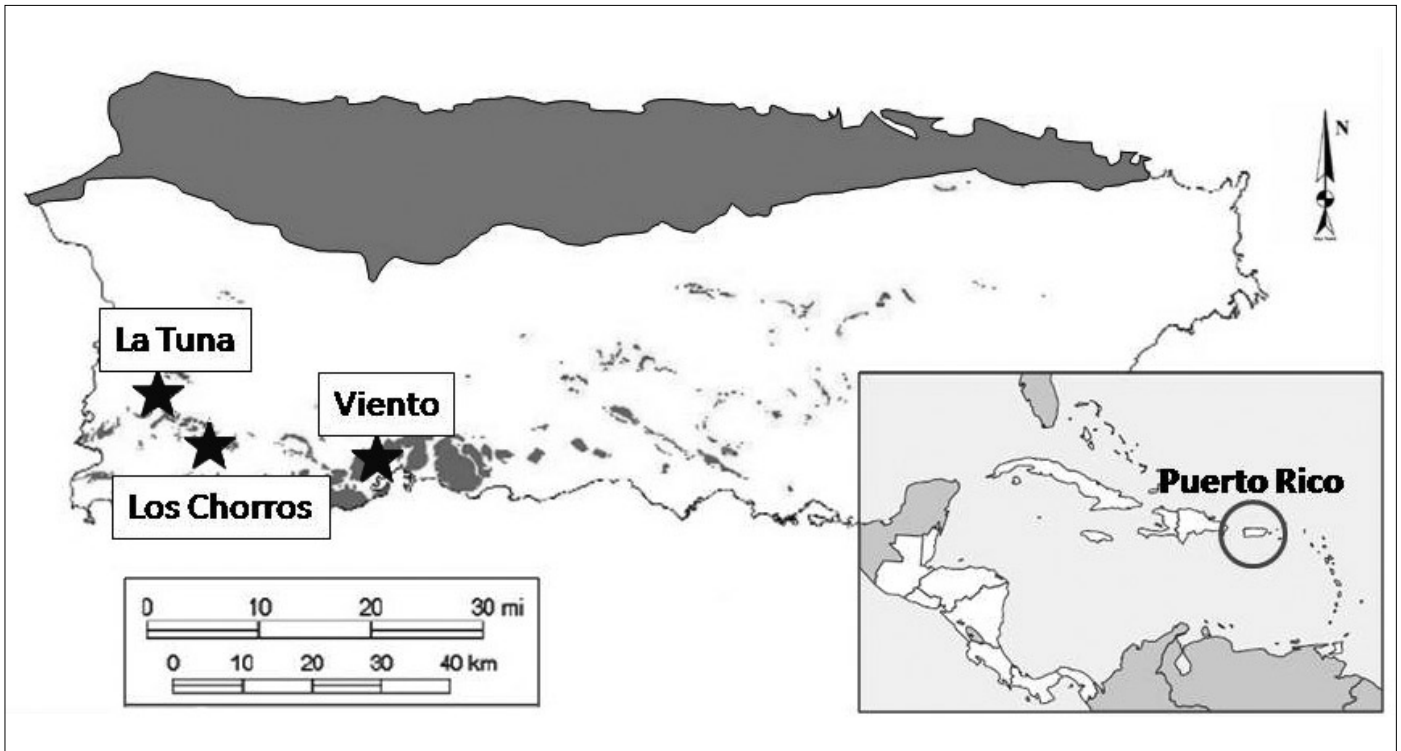


Fig. 1. Puerto Rico and the locations of the three caves discussed in this study (the stars). Gray areas outline the major karsts of the island (after Lugo et al., 2001).

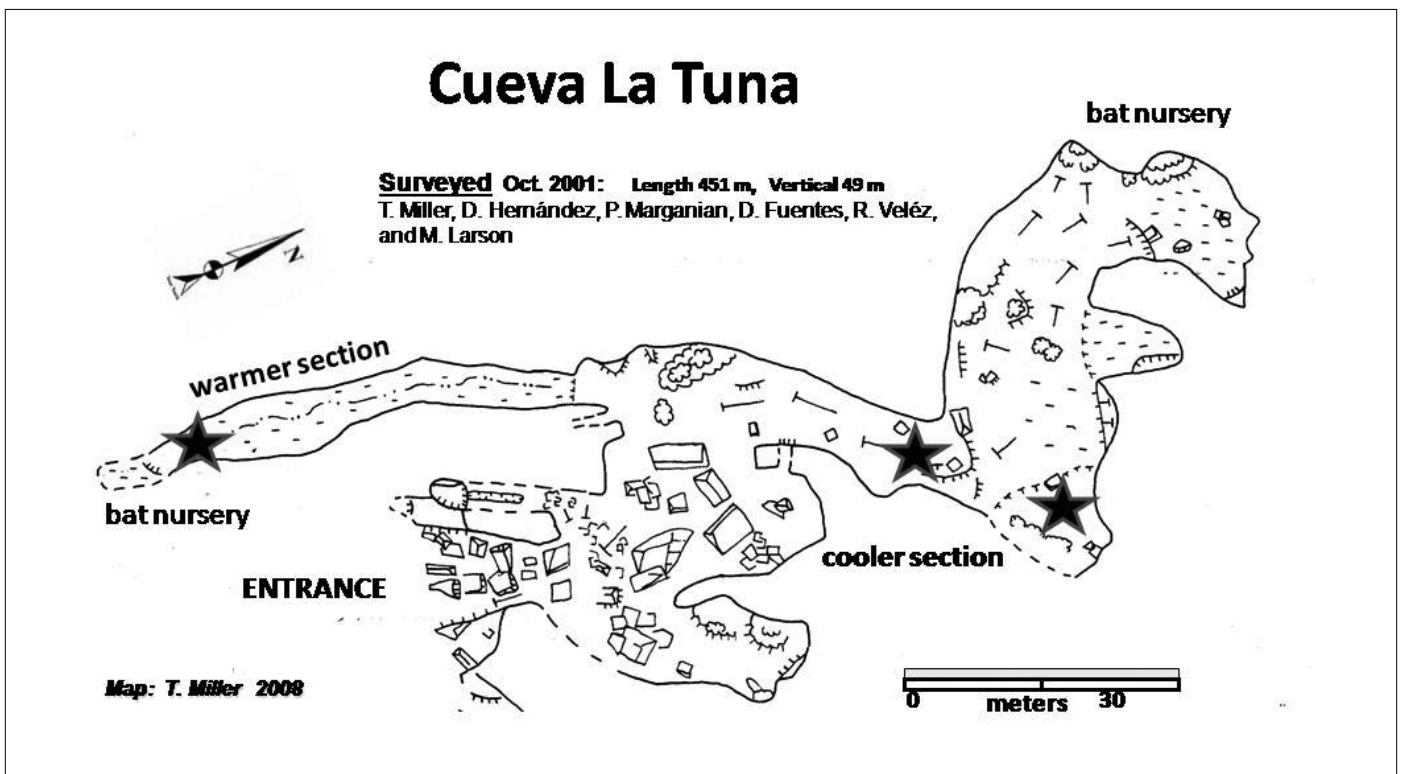


Fig. 2. Cueva La Tuna, approximately 200 m asl. The three sample sites are indicated by the stars. The interior detail uses standard symbols for cave features.

the area with 815 m surveyed. The Sistema de Los Chorros developed at 100-120 m asl in a resistant ridge forming, fault-bounded segment of the Cretaceous Cotuí Limestone (Volckmann, 1984; Miller, 2004). The presence of seeds and etiolated seedlings of *Andira inermis* is notable in several portions of this cave; they were probably carried out into the cave as seeds by bats. These seeds become an ideal substrate for microorganisms, such as fungi.

Like the other two, Cueva Viento (Figure 4) is a hydrological-relict level, lying at least 30 m above the active cave system known as El Convento Cave-Spring System. This cave system is located in the middle of the Tertiary Juana Díaz Formation at the head of a deep, vertically-walled karst gorge known as Quebrada Los Cedros (approximately 5 km ENE of Guayanilla), which is a tributary of the Río Macaná. The underground sections of the stream,

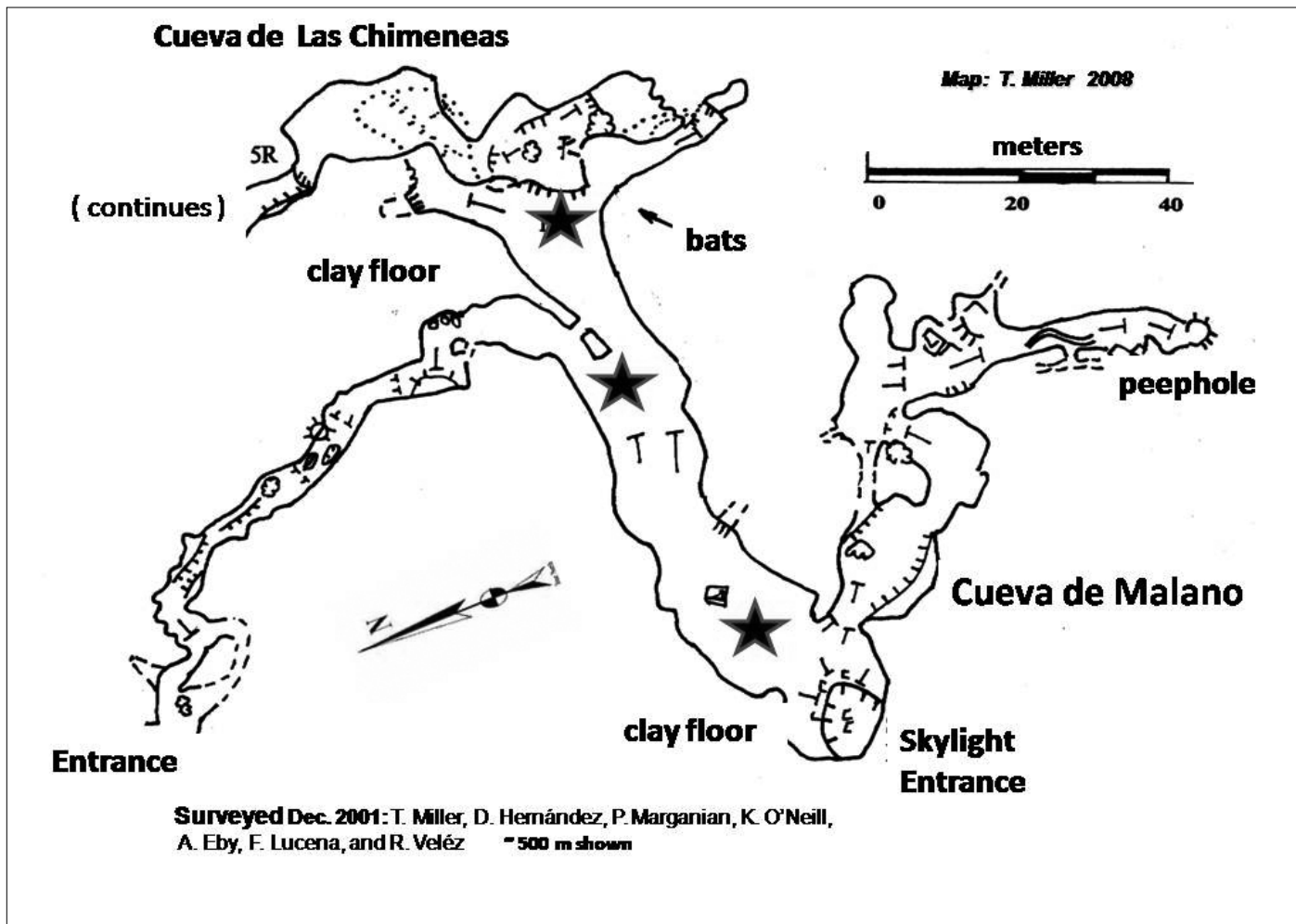


Fig. 3. Cueva de Malano, 100-120 m a.s.l. This is the largest of three sections of Sistema Los Chorros; Cueva de Las Chimeneas is located 15 m vertically above Malano. The stars indicate the three sample sites. From Miller, 2004.

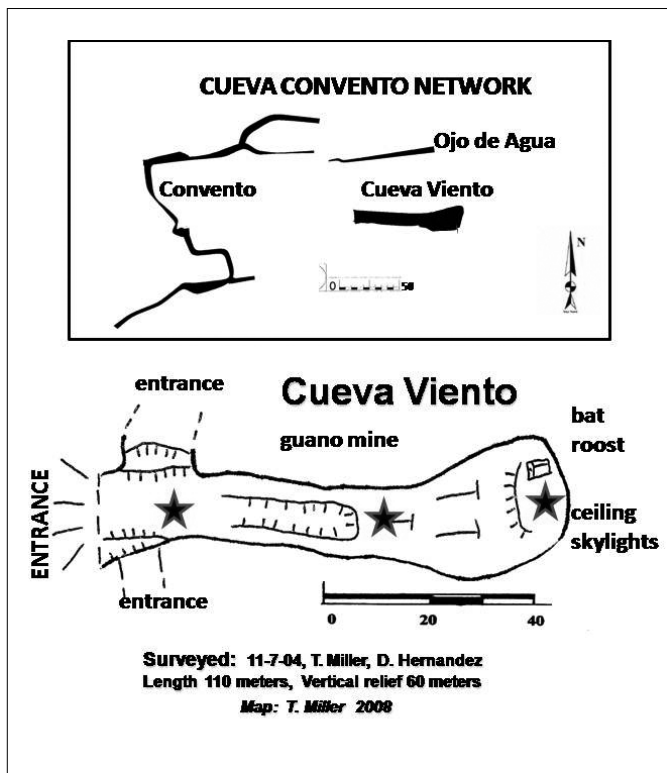


Fig. 4. Cueva Viento (lower figure) 120 m asl; and location relative to other caves in El Convento Cave-Spring System network, after Beck (1974), and Miller (present study). The stars indicate the three sample sites.

cave, and upper level dry cave (Cueva Viento) can be explored for a distance of approximately 500 m (Beck, 1974). It is located in the eastern wall of the Quebrada Los Cedros gorge, approximately 60 m south of the canyon headwall, and is reached via a steep talus slope. Cueva Viento rises almost 40 m in a 20 m wide tunnel, to a large dome, approximately 20 m tall with two skylights (Beck, 1974). A wide excavation about 7 m deep remains from past guano mining. The accumulations of fresh guano indicate a large bat population, especially among the species *A. jamaicensis* and *Brachyphylla cavernarum*, inhabitants of this cave system (Nicholas, 1974). The works of Nicholas (1974) and Peck (1981) documented the vertebrate and invertebrate fauna of this cave system. Cintrón & Beck (1977) have shown how the differing microclimate of this karst gorge has isolated a plant community otherwise typical of subtropical moist forest, within the local dry life zones nearby.

**Collection and Examination of Samples**

For determination of dominant mycobiota, samples of bat guano were placed in glass Petri dishes on filter paper moistened with sterile water and incubated under ambient laboratory conditions until sporulation of fungi was observed under a magnification of 50x (Richardson, 2008). Structures of the predominant fungi were examined at 100-400x. Pieces of wood

associated with bat guano were also collected. Other fungi were isolated from field-collected samples and processed according to Nieves-Rivera (2003), using Potato Dextrose Agar acidified with 10% lactic acid and Rose Bengal Agar as fungal growth media. Wet mount observations were made with a Nikon Labphoto-2 microscope. All specimens were identified by observation of morphological and microscopic characters, along with comparison with specimens from the U.S. National Fungus Collection at Maryland (BPI). Identification of the species of *Circinella* was done according to the scheme of Hesseltine & Fennell (1955) and Zycha et al. (1969). Other taxonomic keys consulted were those of Bell (1983), Seifert et al. (1983), Ellis & Ellis (1988), Richardson & Watling (1997), and Doveri (2004).

## RESULTS AND DISCUSSION

Fifty species of guanophilic fungi were isolated from field-collected samples within the caves sampled; most were mitosporic fungi (23 species) (Table 1;

Figures 5A-F, 6, 7A-B, and 8). The most conspicuous fungus by far was the zygomycete *Circinella umbellata* (Mucorales). *Circinella umbellata* dominated the bat guano incubation chambers. Nineteen species of basidiomycetes (e.g., *Ganoderma* cf. *resinaceum*, *Geastrum* cf. *minimum*, *Lepiota* sp., *Polyporus* sp., *Ramaria* sp.) and three species of ascomycetes (*Hypoxylon* sp., *Xylaria anisopleura*, and *X. kegeliana*) were also recorded. They were found on soil, rotting leaves, bark and rotting wood, buried in bat guano located below natural skylights or sinkholes (Table 1; Figures 5B-D). *Xylaria anisopleura* and *X. kegeliana* have been previously collected from rotten wood in The Ravine of the Río Camuy Caves Park (Nieves-Rivera, unpublished data, 1999).

*Circinella umbellata* is a coprophilous species often recorded from dung of various animals. Zycha et al. (1969) and O'Donnell (1979) provided illustrations and multiple references on this species. Isolates of *C. umbellata* from dog dung, rat dung, lizard dung, and various other types of dung are available from

Table 1. Guanophilic fungi collected in three caves of southwestern Puerto Rico.

Mitosporic fungi	Basidiomycota
<i>Aspergillus flavus</i> Link	<i>Auricularia fuscossuccinea</i> (Mont.) Farl.*
<i>A. fumigatus</i> Fresenius	
<i>A. nidulans</i> (Eidam) G. Winter	
<i>A. niger</i> van Tieghem	<i>A. polytricha</i> (Mont.) Farl.*
<i>Cladosporium cladosporoides</i> (Fresenius) deVries	<i>Clitocybe</i> sp.*
	<i>Collybia aurea</i> (Beeli) Pegler*
<i>C. sphaerospermum</i> Penzig	<i>C. johnstoni</i> (Murrill) Dennis*
<i>C. oxysporum</i> Berk. & M. A. Curtis	<i>Ganoderma</i> cf. <i>resinaceum</i> Boud.*
<i>Curvularia</i> cf. <i>eragrostidis</i> (P. Hennings) Mayer	<i>Geastrum</i> cf. <i>minimum</i> Schw.*
<i>Fusarium oxysporum</i> Schl.	<i>Hexagonia hydnoidea</i> (Sw.) M. Fidalgo*
<i>F. solani</i> (Martius) Sacc.	<i>Lepiota</i> sp.*
<i>Geotrichum</i> sp.	<i>Lepista</i> cf.*
<i>Gliocladium</i> sp.	<i>Mycena</i> sp.*
<i>Hirsutella</i> sp.	<i>Phellinus gilvus</i> (Schw.) Pat.*
<i>Neurospora</i> sp.	<i>Podoscypha</i> sp.*
<i>Paecilomyces</i> sp.	<i>Lepiota</i> sp.*
<i>Penicillium chrysogenum</i> Thom	<i>Polyporus</i> sp.*
	<i>Rigidoporus microporus</i> (Fr.) Overeem.*
<i>P. roqueforti</i> Thom	<i>Ramaria</i> sp.*
<i>Scopulariopsis</i> sp.	<i>Trametes</i> sp.*
<i>Sepedonium</i> cf.	<i>Volvariella</i> sp.*
<i>Trichoderma viridae</i> Pers.: Fr.	<b>Zygomycota</b>
<i>Trichoderma</i> sp.	
<i>Mycelia sterilia</i>	
<b>Ascomycota</b>	<i>Absidia</i> cf.
<i>Hypoxylon</i> sp.*	<i>Circinella umbellata</i> van Tieghem & Le Monnier
<i>Xylaria anisopleura</i> (Mont.) Fr.*	<i>Cunninghamella</i> sp.
<i>X. kegeliana</i> (Lév.) Fr.*	<i>Mucor</i> sp.
	<i>Rhizopus</i> cf. <i>stolonifer</i> (Erh.: Fr.) Vuill.

\* Not coprophilous or guanophilic fungi. Their presence in guano is incidental, because these fungi are saprotrophs (lignicolous, foliicolous) or edaphic, thus we prefer to use the term "guano-associated fungi" for these species.

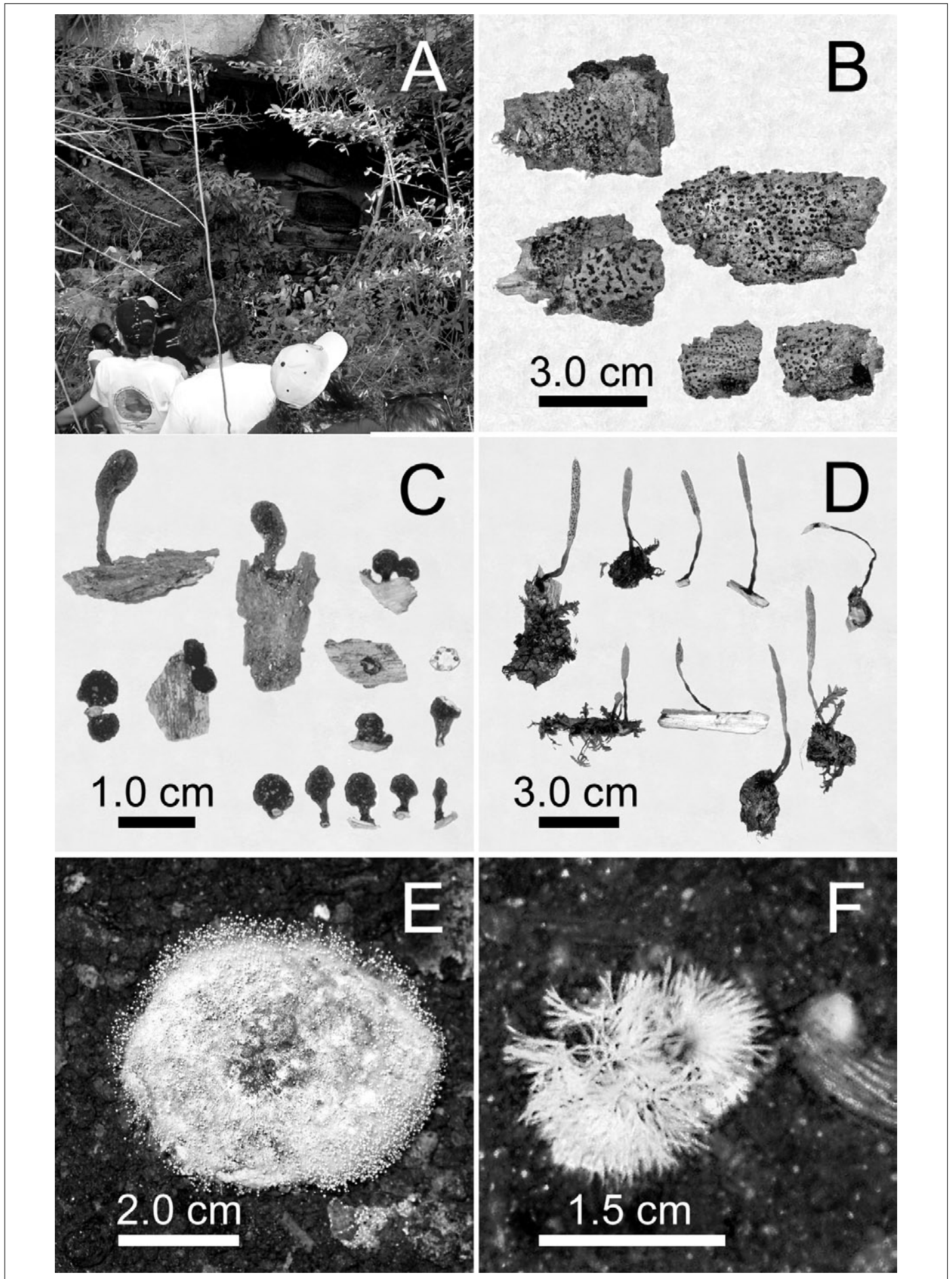


Fig. 5. A-F. Fungi from caves. A. Entrance to Cueva La Tuna. B. *Hypoxylon* sp. on wood. C. *Xylaria anisopleura* on wood. D. *Xylaria kegeliana* on wood. E. *Aspergillus nidulans* on the seed of the Angelin or "Moca" tree (*Andira inermis*) probably brought by roosting bats into the cave (Cueva La Tuna). F. *Mycelia sterilia* on a fecal pellet on bat guano (Cueva de Malano).



Fig. 6. *Mycelia sterilia* on bat guano in Cueva La Catedral (Río Camuy Caves Park, Hatillo, northern Puerto Rico (Nieves-Rivera, 2003).

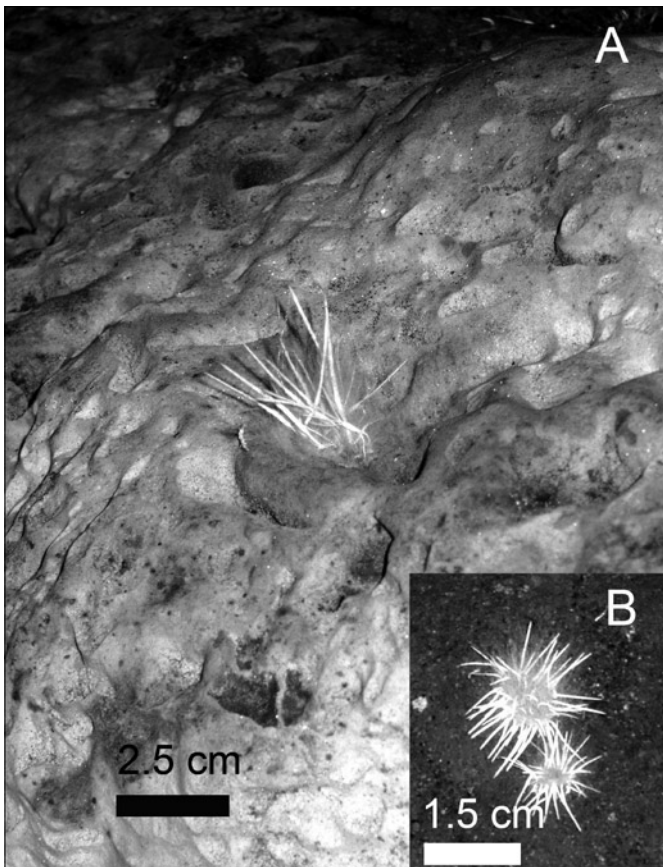


Fig. 7. A-B. *Mycelia sterilia* on stalagmite (A) and fecal pellet on bat guano (B) in Cueva de Malano.

commercial culture collections. We did not observe the sexual stage. Also, *C. umbellata* has been isolated from soil and bat guano of Cueva Las Archillas (Morovis), Cueva La Catedral (Río Camuy Caves Park, Hatillo), Cueva Mata de Plátano or Cueva Los Culebrones (Arecibo), Cueva Matos (Arecibo), and Cueva El

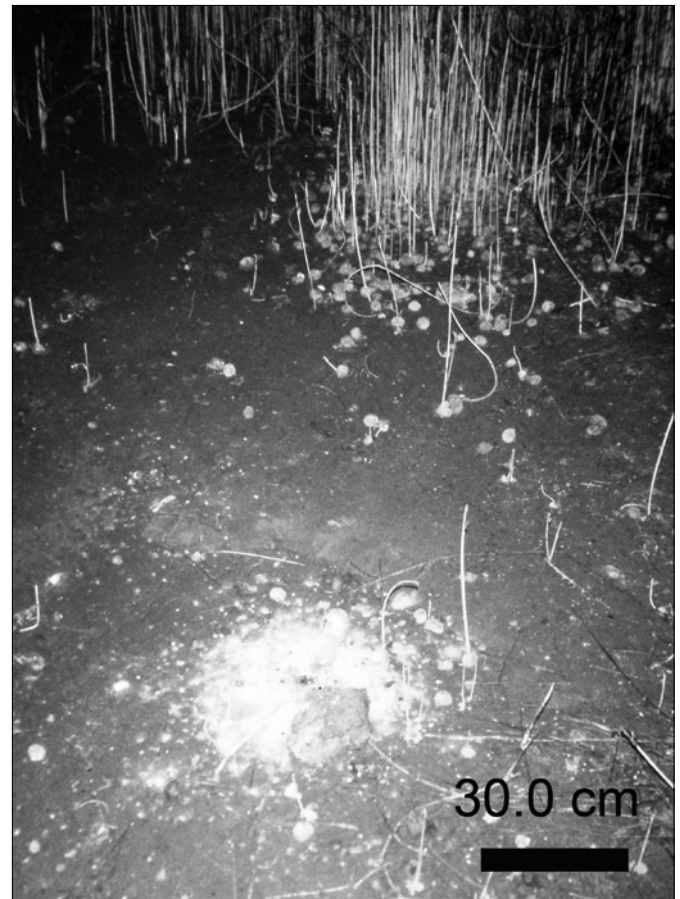


Fig. 8. *Mycelia sterilia* on bat guano in Cueva de Malano. The seeds and etiolated seedlings of *Andira inermis* are notable in several portions of this cave. Photograph was taken by one of us (ÁMNR) during an early exploration on 20 October 1989.

Peñón del Rosario (San Germán). *Circinella umbellata* dominated the fungi in the incubation chambers, but it may not have been the most conspicuous fungus in the cave habitats.

In the present study we did not isolate the fungus *H. capsulatum*. The media (mold inhibitory media not being used) and the methods used for isolation of fungi (no use of mouse-passage technique often employed for recovery of this fungus from samples of bat guano) could account for its non-recovery. One of us (ÁMNR) observed conidia similar to *H. capsulatum* in bat guano in previous surveys but smaller than typical for *H. capsulatum*. The maximum dimension was less than 8  $\mu\text{m}$ , most were between 6-7  $\mu\text{m}$  and there were many smaller ones. Gaur & Lichtwardt (1980), indicated that they screened for conidia of 8-11  $\mu\text{m}$  in diameter, when looking for *H. capsulatum*. An examination of tubercles on conidia obtained in the caves of Puerto Rico showed differences in variability in shape and width in these structures from those of *H. capsulatum* isolates produced in agar culture (L. Sigler, personal communication, 1997). Sigler et al. (1979) and Gaur & Lichtwardt (1980) have demonstrated that similar soil saprobic fungi such as the *Botryotrichum* anamorph of *Chaetomium histoplasmodes*, *Chrysosporium* anamorph of *Renispora flavissima*, *Myceliophthora* anamorph of *Corynascus sepedonium*, and *Sepedonium* produce structures which resemble the tuberculated macroconidia and microconidia of *H. capsulatum*,

making visual identification difficult. Each of these species forms conidia which are generally larger than those of the specimen from Puerto Rico. Isolation of the fungus producing these conidia was unsuccessful (Nieves-Rivera, unpublished data, 1999).

During our expeditions, we have documented six different forms of *Mycelia sterilia* on fecal pellets and bat guano in the caves of Puerto Rico: (1) dendritic or dendroid (like the mycelium of *Collybia johnstoni* on leaf litter) (Figure 5F), (2) acicular to subulate forming the “spiky” or star-like (Figures 6, 7B), (3) filiform (Figure 7A), (4) cottony (Figure 8), (5) foliose (like the mycelium of *Marasmius* spp. on leaf litter), and (6) combination of two or more forms. In Figures 5F, 6, 7A-B, and 8, we showed four of the six different forms of *M. sterilia*. The forms of *M. sterilia* illustrated include a hyaline “spiky” or star-like, acicular to subulate, filiform with tapered base, mostly straight, having a dehiscent appearance, about 1 to 6 cm in diameter, sometimes larger. Since no fruiting structures or spores have been detected, we tentatively named them *M. sterilia*. In Cueva de Malano, *M. sterilia* was incidentally found on a moist calcite stalagmite (Figure 7A), but mostly on the ground and under a bat roost (Figure 7B). Fungi have been isolated from the surface water film of limestone and calcite speleothems elsewhere (Hasselbring et al., 1975).

It has been suggested that many of the fungal-like structures that have been identified as *M. sterilia* in this study are actually aragonite helictites. Aragonite helictites are specialized speleothems in limestone caves that occur as a result of seepage through a central pore (0.2-0.4 mm in diameter) and deposition at the tip, forming eccentrics that defy gravity. Although *M. sterilia* might resemble aragonite helictites (see lower illustration of page 57 in Moore & Sullivan, 1978), a closer examination might reveal their fungal origin. There are certain differences to be noticed; for instance, there is no central pore, they are composed of mycelium (mostly coenocytic but sometimes septate), and they occur in association with fecal pellets, guano, seeds, and fruits.

Basidiomycetes were also found on various substrates (on soil, rotting leaves, bark and rotting wood buried in bat guano located below natural skylights or sinkholes and through the gorge of the El Convento Spring-Cave System during the course of our expeditions (Table 1). These basidiomycetes occurred at the entrance of caves or throughout the gorge per se. Most of these species are new records for this karstic isolated zone, however they are commonly found in the forests of Puerto Rico (Lodge, 1996; Minter et al., 2001; Cantrell et al., 2006; Cantrell & Lodge, 2008).

Fungal species collected from the sites considered in the present survey were similar to those found in previous surveys (Carvajal-Zamora & Nieves-Rivera, 1998; Nieves-Rivera & Carvajal-Zamora, 2000; Nieves-Rivera, 2003; Pedro & Bononi, 2007). Many of the species collected are common saprobes of soil, dung, and plant debris. In deep caves, the long isolation from the surface input may cause food deprivation, starvation or low nutrient for the fungal

population. However, bat guano is a rich substrate, which combined with high humidity along with stable temperatures through the year, provides optimal environmental conditions (a natural Petri dish), ideal for fungal development.

The importance of studying cave fungi relates to the fact that many species have the ability to produce mycotoxins that repel microorganisms (e.g., bacteria, protists, nematodes). Fungi also recycle the nutrients in the caves; therefore they constitute a crucial link to the trophic chain. The possibility of novel discoveries is not exhausted and, as suggested in Lodge (1996), it would be a monumental task to produce a reasonably complete mycobiota for Puerto Rico. Therefore, it seems important to continue studying such habitats, in order to contribute to the conservation and knowledge of the speleo-biodiversity of Puerto Rico.

### ACKNOWLEDGEMENTS

We would like to dedicate this work to the memory of the Nicaraguan biospeleologist and mycologist Juan Ramón Carvajal-Zamora (1944-2006), who in September 2006 passed away at the age of 61, after a fruitful life of research and being one of the founding fathers of Puerto Rican biospeleology and limnology. In addition, we thank María Ruiz-Yantín (Dept. of Earth and Environmental Sciences, Vanderbilt University, Nashville) for her help to this study. Thanks to Lynne Sigler (Microfungus Collection and Herbarium, University of Alberta, Edmonton, Canada), Barry F. Beck (P.E. LaMoreaux & Associates, Inc., Oak Ridge), Stewart B. Peck (Dept. of Biology, Carleton University, Ottawa, Canada), and Steven L. Stephenson (Dept. of Biological Sciences, University of Arkansas, Fayetteville) for their comments or review of the manuscript. We thank Jack D. Rogers (Dept. of Plant Pathology, Washington State University, Pullman) for the identification of the *Xylaria* species. Figures 5A-F was prepared by Peter Rocafort (Dept. of Marine Sciences, University of Puerto Rico, Mayagüez) and Figure 6 was kindly provided by Luis E. Collazo (Dept. of Crop Protection, University of Puerto Rico, Mayagüez).

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