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REPORT

Fungi in healthy and diseased sea fans (*Gorgonia ventalina*): is *Aspergillus sydowii* always the pathogen?

C. Toledo-Hernández · A. Zuluaga-Montero · A. Bones-González · J. A. Rodríguez · A. M. Sabat · P. Bayman

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Abstract Caribbean corals, including sea fans (*Gorgonia* spp.), are being affected by severe and apparently new diseases. In the case of sea fans, the pathogen is reported to be the fungus *Aspergillus sydowii*, and the disease is named aspergillosis. In order to understand coral diseases and pathogens, knowledge of the microbes associated with healthy corals is also necessary. In this study the fungal community of healthy *Gorgonia ventalina* colonies was contrasted with that of diseased colonies. In addition, the fungal community of healthy and diseased tissue within colonies with aspergillosis was contrasted. Fungi were isolated from healthy and diseased fans from 15 reefs around Puerto Rico, and identified by sequencing the nuclear ribosomal ITS region and by morphology. Thirty fungal species belonging to 15 genera were isolated from 203 *G. ventalina* colonies. *Penicillum* and *Aspergillus* were the most common genera isolated from both healthy and diseased fans. However, the fungal community of healthy fans was distinct and more diverse than that of diseased ones. Within diseased fans, fungal communities from diseased tissues were distinct and more diverse than from healthy tissue. The reduction of fungi in diseased colonies may occur prior to infection due to environmental changes affecting the host, or after infection due to increase in dominance of the pathogen, or because of host responses to infection. Data also indicate that the fungal community of an entire sea fan colony is affected even when only a small portion of the colony suffers from aspergillosis. An unexpected result was that *A. sydowii* was found in healthy sea fans but never in diseased ones. This result suggests that *A. sydowii* is not the pathogen causing aspergillosis in the studied colonies, and suggests several fungi common to healthy and diseased colonies as opportunistic pathogens. Given that it is not clear that *Aspergillus* is the sole pathogen, calling this disease aspergillosis is an oversimplification at best.

Keywords *Gorgonia ventalina* · *Aspergillus sydowii* · Aspergillosis · Coral diseases

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C. Toledo-Hernández (✉) · A. Zuluaga-Montero · A. Bones-González · J. A. Rodríguez · A. M. Sabat · P. Bayman
Department of Biology, University of Puerto Rico-Río Piedras, P.O. Box 23360, San Juan 00931, Puerto Rico
e-mail: c_toledo_hernandez@yahoo.com

Introduction

An alarming increase in diseases of corals has been reported in the last twenty years (Gardner et al. 2003). Since corals are the main structural components of coral reefs, these diseases may threaten this ecosystem in general (Bellwood et al. 2004). Of the coral diseases reported thus far, aspergillosis of sea fans is one of the best understood. This disease was first reported as causing tissue mortality in the Caribbean sea fans *Gorgonia ventalina* and *Gorgonia flabellum* (Nagelkerken et al. 1997). *Aspergillus sydowii* was identified as the causative agent of aspergillosis based on sequences of the 18S nuclear ribosomal gene, morphology, and Koch's postulates (Smith et al. 1996; Geiser et al. 1998). Three sources of inoculum have been proposed: (1) spores or hyphae of *A. sydowii* associated with terrestrial runoff or airborne dust; (2) physical contact between healthy and diseased sea fans; and (3) waterborne infection of healthy colonies by hyphae or spores from an infected colony (Jolles et al. 2002). Incidence of sea fan aspergillosis has been related, in part, to a reduction in host defense and to an enhancement of fungal growth as a result of increases in water temperature (Alker et al. 2001; Dube

et al. 2002; Kim and Harvell 2004). Sea fans aspergillosis is also characterized by high temporal and spatial variability (Kim and Harvell 2004), which complicates its study.

The sea fan aspergillosis literature states or assumes that *A. sydowii* is the responsible pathogen, as reported by Smith et al. (1996) and Geiser et al. (1998). However, lack of knowledge of the fungal community associated with healthy sea fans undermines an understanding of the roles of fungi when colonies become diseased. Recently, *A. sydowii* and other potentially pathogenic fungi were isolated from *G. ventalina* colonies without signs of aspergillosis (Toledo-Hernández et al. 2007). These findings highlight our ignorance of the basic microbial ecology of sea fans.

This study characterizes the fungal community associated with healthy and diseased *G. ventalina* colonies to address the following questions. Is the fungal community of healthy *G. ventalina* colonies different in diversity and composition to that of diseased ones? Within colonies with aspergillosis, does the fungal community of healthy tissue differ from that of diseased tissue? Is *A. sydowii* part of the resident mycoflora of healthy sea fan colonies? Can aspergillosis also be caused by other species of *Aspergillus*, or other fungi?

Materials and methods

Isolation and identification of fungi

Two hundred and three *G. ventalina* colonies from 13 reefs distributed around Puerto Rico were surveyed between 2004–06 (Fig. 1). The number of colonies surveyed per reef ranged between 12 and 20. Of the 203 colonies surveyed, 122 were healthy colonies (i.e., no lesions, no purpling, nor any tissue overgrowth by fouling organisms), while 81 colonies showed signs of aspergillosis (necrotic area surrounded by a purple halo, as defined by Petes et al. 2003). Colonies were selected within an area of at least 1,500 m^2 in each reef taking care not to select close neighbors. Diseased colonies were actively searched for, as they were relatively rare. The size of surveyed sea fans was 900–2,000 cm^2. Since diseased colonies <900 cm^2 were not observed, healthy colonies smaller than this were also excluded. One tissue sample of 2 × 2 cm was collected from each healthy colony while 2 tissue samples of 2 × 2 cm were collected from the diseased colonies, one from a healthy area and the other from an aspergillosis lesion. Tissue samples were immediately placed in individual sterile 50 ml tubes filled with filtered, autoclaved seawater and transported in a cooler with ice for processing within 24 h of collection. To eliminate fungi present on the surface of fan fragments but not colonizing internal tissues, each fragment was surface-sterilized individually in ethanol 70% for 30 s and then washed in filtered, autoclaved seawater for another 30 s. Sodium hypochlorite was not used for sterilization because it dissolves the mesoglea, and may kill some fungi (Koh et al. 2000). Fragments were plated on Glucose Peptone Yeast Agar (GPYA), a standard medium for marine fungi (3 g glucose, 0.3 g yeast extract, 0.3 g peptone, and 20 g agar l^{-1} filtered seawater), and incubated at 28°C in the dark for one month or until fungi were observed (Toledo-Hernández et al. 2007). Fungi were isolated in pure culture and (where possible) identified by morphology. However, some colonies did not sporulate and morphology-based identification was thus not possible; otherwise, identifications based on DNA sequence data agreed with morphology. Representative strains of each morphospecies were chosen for DNA extraction. For DNA extraction, emerging fungi were transferred to liquid Potato Dextrose (PD) medium made with filtered, autoclaved seawater. (PDA is more nutrient-rich than GPYA; it is less useful as an isolation medium because it encourages the growth of bacteria, but extracts grown on PDA yield more DNA than GPYA.) After five days, DNA was extracted using a Plant Mini Extraction Kit (Qiagen Sciences). The nuclear ribosomal ITS region was amplified using primers ITS1F and ITS4 and annealing temperature of 52–56°C (White et al. 1990) and sequenced. Sequences were corrected using Sequencher and the most similar sequences in GenBank were found using BLAST searches. When the top three matching BLAST hits were from the same species and ≥95% similar to the query sequence, this name was assigned to the culture.

Data analysis

Estimates of the number of fungal species in healthy tissue from healthy colonies (HH), and healthy and diseased tissues from diseased colonies (HD and DD, respectively) were made using the Chao2 and Jackknife1 estimators in EstimateS 8.0.0 (Colwell 2006). A richness/species abundance coefficient Bray-Curtis (S_{ab}) was estimated based on presence/absence matrix of fungi isolated from the three tissue types. An analysis of similarity (ANOSIM) and the contribution of each species to the average Bray-Curtis dissimilarity among the three tissue types (SIMPER) were performed using the statistical software PRIMER for Windows version 5.2.9. For this analysis 129 samples were used (no fungi were isolated from the other 74 colonies).

Results

Fungal diversity

Fifteen fungal genera, 30 species, and one unknown isolate with affinities to the Helotiaceae were isolated from the

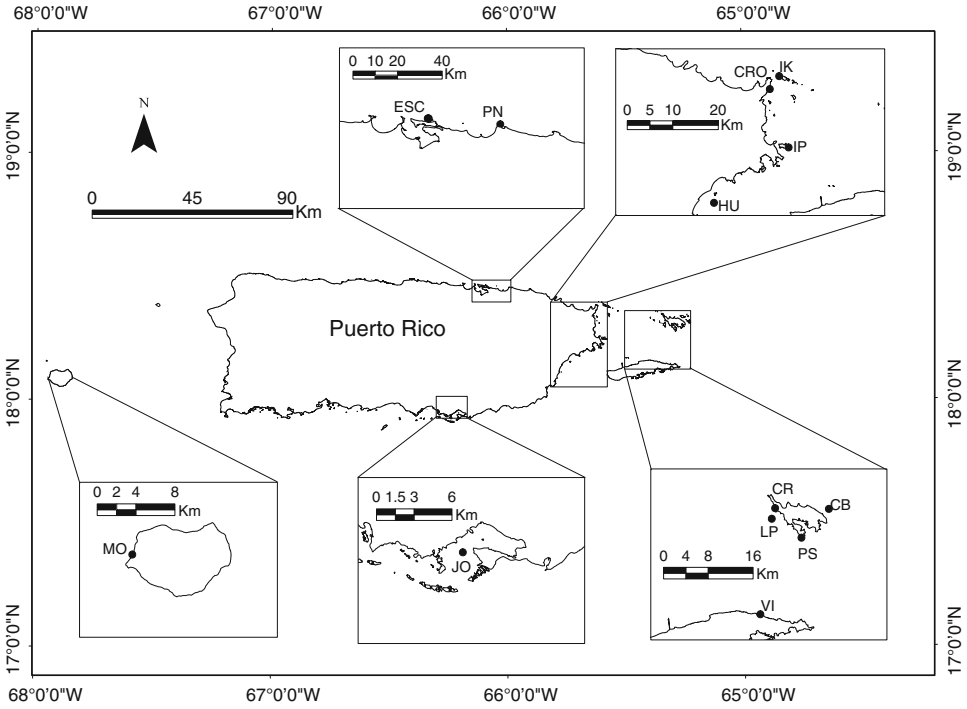

Fig. 1 Map of Puerto Rico showing collection sites. LP Luis Peña, CR Carlos Rosario, CB Culebrita, PT Punta Soldado, VI Vieques Island, ESC Escambrón, PN Piñones, IK Icacos, IP Isla Piñero, HU Humacao, JO Jobos, MO Mona Island

203 *G. ventalina* colonies surveyed (Table 1). *Aspergillus* was the most common and the most diverse genus with 12 species and 64 isolates (Table 1). The second most common genus was *Penicillium* with 5 species and 33 isolates. Most of the remaining genera occurred as singletons and doubletons. Twenty-two taxa are new reports for *G. ventalina* and gorgonian corals in general: *Aspergillus aculeatus*, *Aspergillus melleus, Aspergillus ochraceus*, *Aspergillus tamarii*, *Aspergillus terreus*, *Aspergillus versicolor, Candida* sp., *Chalaropsis* sp., *Cladosporium cladosporioides*, Helotiaceae, *Hypocrea lixii*, *Nectria haematococca*, *Nectria gliocladioides*, *Penicillium chrysogenum*, *Penicillium minioluteum*, *Pichia guilliermondii*, *Stachybotrys chlorohalonata*, *Trichoderma harzianum*, and *Tritirachium* spp. For some fungi, identification inferred from BLAST searches could not distinguish between closely related species. This could reflect limitations of the sequences currently available in GenBank or taxonomic problems in certain species groups (i.e., *Aspergillus flavus* vs. *Aspergillus oryzae* (Geiser et al. 1998, 2000).

Variation in fungal community structure among tissue types

Species richness varied among the three tissue types. HH (healthy tissue from healthy colonies) was highest (25 taxa and 88 isolates, 6 of which could not be identified) followed by DD (diseased tissue from diseased colonies, 15 taxa and 32 isolates, 13 of which could not be identified) and HD (healthy tissue from diseased colonies, 15 taxa and 22 isolates, 6 of which could not be identified) (Table 1). Although no asymptote was reached in any tissue type, the Chao2 and Jackknife 1 indexes predicted the highest species richness for HH (Fig. 2). This suggests that the highest fungal species richness in HH was not due to greater sampling effort, but to a natural pattern.

The most common fungi differed among tissue types. *A. flavus*, *A. sydowii*, and *Penicillium citrinum* were the most frequently isolated fungi in HH (Table 1; Fig. 3). *A. sydowii*, was only isolated from healthy colonies, contrary to expectations. In contrast, *A. flavus* was the most common fungus in HD followed by *C. cladosporoides* (Table 1; Fig. 3). In DD, *A. flavus*, *P. citrinum*, and *Tritirachium* spp. were the most frequently isolated fungi (Table 1; Fig. 3). Of all the isolated fungi, 5 were common to all tissue types, 3 were shared exclusively between HH and HD, 6 were shared exclusively between HH and DD, while no fungi were shared exclusively between HD and DD (Table 1). Moreover, 9 fungi were exclusively isolated from HH (including *A. sydowii*), 7 were exclusively isolated from HD, and 4 fungi from DD (Table 1, Fig. 3). The ANOSIM test showed significant differences in fungal communities among tissue types ($r = 0.034$; $P = 0.002$). The SIMPER analysis showed 93.6% dissimilarity between HH and HD, 90% dissimilarity between HH and DD, and 91.3% dissimilarity between HD and DD. HH was distinguished from HD and DD by the

Table 1 Fungi isolated from *Gorgonia ventalina* as identified by ITS sequences

Closest match in GenBank	Number of isolates per tissue type			Range Max ID %	Range bp sequenced	GenBank accession no.
	HH	HD	DD			
Aspergillus aculeatus	2	1	0	99	542	EU554629
Aspergillus flavus/oryzae	14	5	9	97–100	499–597	EU554573
Aspergillus melleus[a]	1	0	0			
Aspergillus niger	3	0	2	99	503–518	EU554625
Aspergillus. niger/foetidus/awamori	0	1	0	100	1,127	EU554626
Aspergillus ochraceus[a]	0	0	1			
Aspergillus sydowii	10	0	0	100	519–550	EU554604
Aspergillus tamarii	2	0	1	100	559	EU554619
Aspergillus terreus	3	2	1	100	549	EU558539
Aspergillus unguis	1	0	0	99	595	EU554612
Aspergillus ustus	4	1	0	99	519	EU554620
Aspergillus versicolor	2	0	1	99–100	450–525	EU554616
Candida sp.	2	0	0	100	747	EU554623
Chalaropsis sp.	1	0	0	99	446	EU554628
Cladosporium sp.[a]	0	0	1			
Cladosporium cladosporioides	3	3	1	99	538	EU554601
Cladosporium spaerosperma	0	1	0	99	509	EU554622
Davidiella tassiana	2	0	0	99–100	514–538	EU554613
Gloeotinia temulenta	4	1	2	99–100	453–574	EU554591
Helotiaceae	0	1	0	98	491	EU554571
Hypocrea lixii	0	0	1	99	520	EU554589
Nectria sp./*Bionectria*	0	0	1	90	520	EU554570
Nectria gliocladioides	1	0	1	98–99	512–516	EU554567
Nectria haematococca	0	1	0	82	520	EU554568
Penicillium chrysogenum	2	0	0	100	549	EU554618
Penicillium citreonigrum	0	1	0	99	578	EU558538
Penicillium commune	0	1	0	100	534	EU554627
Penicillium minioluteum	1	0	1	99–99	537–529	EU554569
Penicillum citrinum	23	1	4	99–100	219–543	EU554607
Pichia guilliermondii	1	0	0	98	536	EU554590
Rhodotorula nymphaeae	1	0	0	99	530	EU558535
Stachybotrys chartarum	1	0	0	99	433	EU554595
Stachybotrys chlorohalonata	0	1	0	99	491	EU554624
Trichoderma harzianum[a]	3	1	0			
Tritirachium spp.	1	0	4	95–99	509	EU554617
Xylaria hypoxylon	0	0	1	99	563	EU558536
Unknown[b]	6	5	13			
Totals	94	27	45			

Three tissue types were used for isolations: HH, healthy tissue from healthy *G. ventalina* colonies; HD, healthy tissue; DD, diseased tissue, from diseased colonies. Range of maximum identity of the closest three matches listed in GenBank (Max ID %); number of base pairs sequences (bp); GenBank accession numbers of isolates from *G. ventalina*

[a] Identified on the basis of morphology alone

[b] Fungi were lost before they could be sequenced or identified by morphology

greater relative abundance of *A. flavus, A. sydowii*, and *P. citrinum* whereas HD was distinguished from DD by the greater relative abundance of *A. flavus, P. citrinum*, and *C. cladosporiodes*. Only one fungus was more common in diseased tissue than in other tissue types: *Tritirachium* sp. (Table 1).

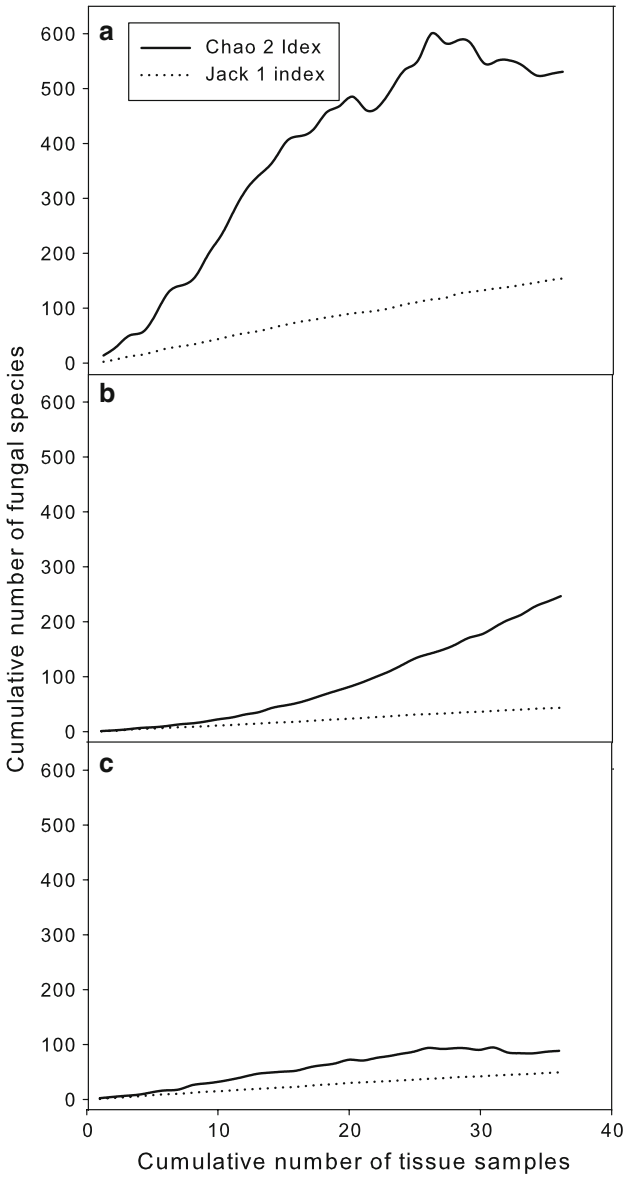

Fig. 2 Species accumulation curves of Chao2 and Jackknife1 estimators for fungi isolated from healthy tissue of (**a**) healthy *Gorgonia ventalina* colonies, (**b**) healthy tissue, and (**c**) diseased tissue of diseased colonies

Discussion

Fungal diversity associated with gorgonians

This study, in conjunction with those of Koh et al. (2000) and Toledo-Hernández et al. (2007), shows that sea fans have a diverse and variable fungal community. Overall, 65 fungal taxa have been identified from 11 gorgonian species using culture-dependent techniques. Sixteen fungal genera and 51 species (including two yeasts) were isolated from 10 species of gorgonian corals in Singapore (Koh et al. 2000). All of these fungi were new reports for gorgonians. Eight genera of fungi and 15 species were isolated from the Caribbean sea fan *G. ventalina* (Toledo-Hernández et al. 2007). Four of these genera, *Rhodotorula*, *Stachybotrys*, *Gloeotinia*, and *Xylaria,* and an unknown fungus with affinities to the Xylariales were new reports for any gorgonian coral. In this study, at least 35 fungal species from 15 genera were identified from 203 *G. ventalina* colonies. Twenty-three of these species are new reports for *G. ventalina* or any other gorgonian coral. Here as well as in previous studies (Koh et al. 2000; Toledo-Hernández et al. 2007), *Aspergillus* and *Penicillium* were the most frequently isolated and diverse genera. *Aspergillus* and *Penicillium* also appear to be common in other marine invertebrates such as scleractinian corals (Kendrick et al. 1982; Priess et al. 2000) and sponges (Höller et al. 2000). Some of these species (e.g., *A. sydowii*) are believed to be essentially terrestrial organisms, capable of growing in the sea but incapable of sporulating there (Smith et al. 1996). However, their presence in many marine invertebrates suggests they are ubiquitous in the marine environment.

Differences in fungal diversity between healthy and diseased sea fans

This study shows that (1) the fungal community of healthy sea fans is distinct from, and more diverse than, that of diseased fans and (2) within afflicted colonies the fungal community of lesions is different and more diverse than that of healthy tissue. Previous studies have reported differences in bacterial communities between healthy and diseased tissue from diseased scleractinian corals (Frías-López et al. 2002; Casas et al. 2004; Breitbart et al. 2005). However, these studies did not include healthy colonies, which were found here to be distinct from healthy tissue in diseased colonies. Only one previous study has contrasted the microbial community between healthy and diseased colonies as well as between healthy and afflicted tissue within diseased colonies (Pantos et al. 2003). Bacterial communities in *Montastraea annularis* followed the same pattern observed here for fungi in *G. ventalina*: healthy tissue in healthy colonies was most diverse, healthy tissue from diseased colonies was least diverse, and diseased tissue was intermediate (Pantos et al. 2003). There are three plausible explanations why healthy tissue from diseased colonies showed decreased microbial richness compared to diseased tissue. First, the reduction of microbial diversity in diseased colonies may be directly caused by an increase in dominance of the pathogen (Ward et al. 2007). Second, environmental changes may reduce microbial diversity, making the host more susceptible to pathogens (Pantos et al. 2003). Third, the colony may have a generalized physiological response to a local infection, which could affect commensal microorganisms as well as pathogens. Similarly, localized infections

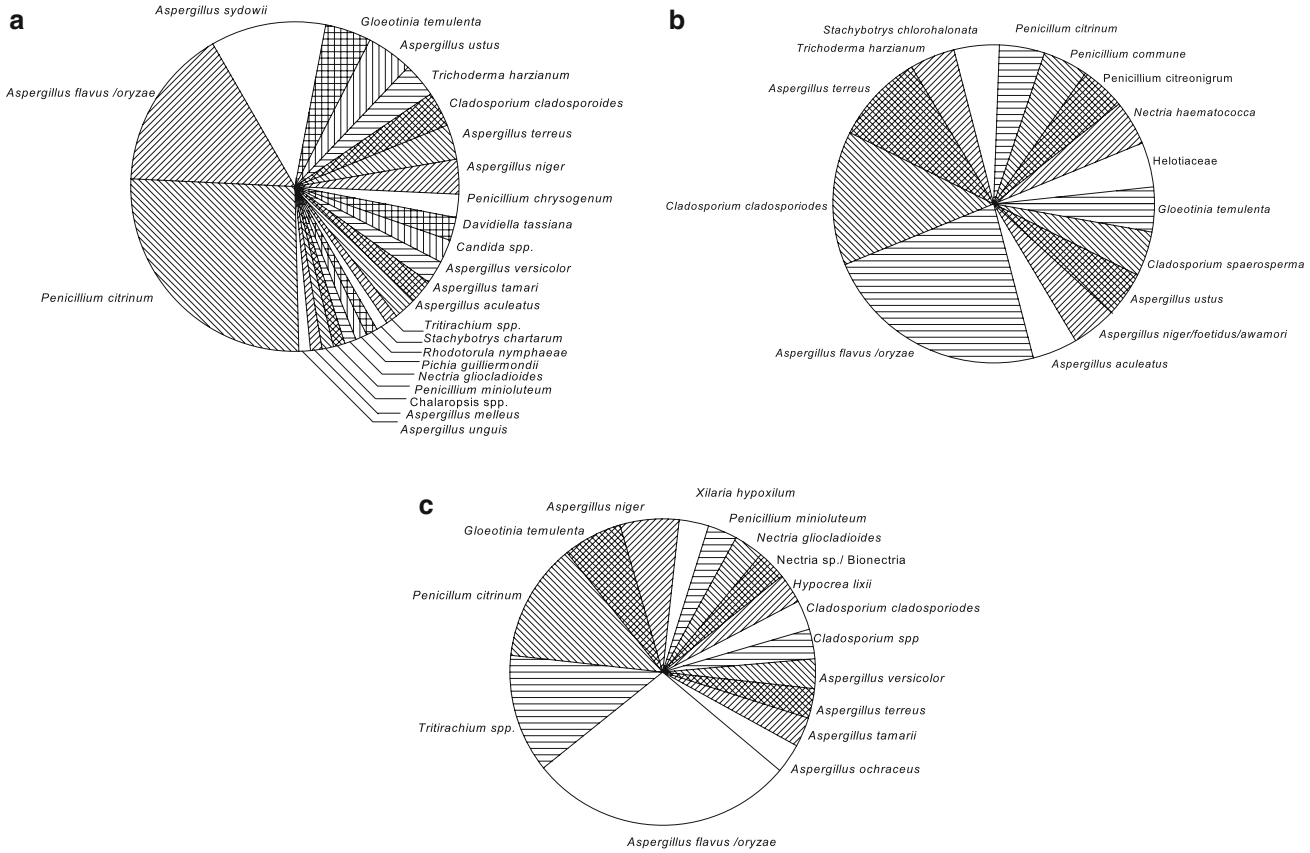

Fig. 3 Pie diagram illustrating the observed frequencies in percentages of fungi isolated from healthy tissue from (**a**) healthy *Gorgonia ventalina* colonies, (**b**) healthy tissue, and (**c**) diseased tissue from diseased colonies

have a general effect on reproduction: even small lesions affect reproductive success of the whole colony (Petes et al. 2003).

Is *A. sydowii* a pathogen?

The most striking result of this study is that *A. sydowii*, the reported causal agent of sea fan aspergillosis, was not isolated from diseased *G. ventalina* colonies, but only from healthy colonies. These observations cast doubt on the role of *A. sydowii* as the pathogen (either primary or opportunistic) of sea fan aspergillosis, at least in the colonies studied. The most common isolates (e.g., *A. flavus* and *P. citrinum*) were found in both healthy and diseased colonies, suggesting they may be opportunistic pathogens capable of causing aspergillosis.

These two species are prime suspects. *A. flavus* is an opportunistic pathogen of insects, bird, humans, and plants (Yu et al. 2005) and produces secondary metabolites called aflatoxins that are toxic, carcinogenic, teratogenic, and immunosuppressive in animals when ingested (Pitt 2000). *P. citrinum* produces the toxin citrinin, associated with renal toxicity in animals (Malmstrom et al. 2000). It is not known if any of these mycotoxins are produced in vivo in sea fans, but if they are, it is possible they play a role in the disease.

Another possible pathogen is *Tritirachium* sp., the only species that was more common in diseased tissue than in other tissue types. *Tritirachium* has not been reported from corals previously, but based on phylogeny, this fungus might be expected to be a pathogen: it belongs to the Clavicipitaceae, all members of which are obligate pathogens of invertebrates or plants.

Alternatively, sea fan aspergillosis may be caused by pathogens other than fungi. However, little or no effort has been devoted to study the bacteria, protists, and viruses associated with sea fans, perhaps because *A. sydowii* is widely assumed to be the primary pathogen. Controlled infection experiments are needed to test the pathogenicity of fungi and other microorganisms found in sea fans.

Methodology and sampling issues

In this study, fungi were cultured before being identified, so any fungi that did not grow in culture were overlooked. Estimates of diversity presented here therefore under-represent total fungal diversity, though it is impossible to say by how much. Studies comparing culturable and nonculturable

fungi in other communities suggest that nonculturable species are a much smaller percentage of total diversity for fungi than for bacteria. In fungal endophytes of pine, for example, culturing revealed more major groups of fungi than direct amplification, and was more effective at revealing species richness of certain groups of fungi (Arnold et al. 2007). Since each technique revealed some organisms not revealed by the other, a combination of direct amplification and culturing is the optimal approach for fungal diversity studies (Rohwer et al. 2001; Bayman 2007). Direct amplification of fungi and other microorganisms from sea fan tissue would clearly reveal more species. Nevertheless, this limitation does not affect the most important conclusion of this study, that regarding *A. sydowii*. Like most species of *Aspergillus*, *A. sydowii* grows readily in culture under a wide range of conditions. If anything, culture-based studies may overstate the importance of *Aspergillus* and *Penicillium* relative to fungi that do not grow so rapidly and prolifically on agar media.

The present study demonstrates that *G. ventalina* colonies contain a large, diverse, and mostly unknown fungal community. It also shows that the fungal community of an entire sea fan colony is affected even when only a relatively small portion of the colony suffers from aspergillosis; the fact that this pattern was also observed in bacterial communities in scleractinian corals (Pantos et al. 2003) suggests that it may be a general phenomenon, though the cause is unclear. This study did not find *A. sydowii* associated with diseased colonies, suggesting that *A. sydowii* is not the pathogen, at least in Puerto Rico. The presence of several common fungi in both diseased and healthy colonies suggests that aspergillosis may not always be a result of the arrival of new pathogens, but a change in population size or pathogenicity of fungi already present. The importance of environmental stress in the development of aspergillosis has been discussed (Lesser et al. 2007). However, population dynamics of microorganisms in corals and the interaction between microbial communities and stress are not understood.

Initial reports that *A. sydowii* was the pathogen responsible for aspergillosis in *Gorgonia* were no doubt accurate (Smith et al. 1996; Geiser et al. 1998); they reflected pathogens of gorgonians in certain areas at certain times. However, subsequent articles generalized these results to mean that *A. sydowii* was the cause of aspergillosis of gorgonians throughout the Caribbean, which is clearly not the case. Given that it is not clear that *Aspergillus,* or any fungus, is the sole pathogen, calling this disease aspergillosis is an oversimplification. Aspergillosis of sea fans has been viewed as one of the best-understood coral diseases; the fact that it turns out to be more complicated than previously thought implies that other coral diseases may be so as well.

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