Fungi associated with gorgonians in Singapore

L.L. Koh[1], T.K. Tan L.M. Chou [1]and N.K.C. Gob[1]

ABSTRACT

The aims of this preliminary study were: I) to document the diversity of fungi associated with gorgonians near Singapore, 2) to determine whether the kinds and abundances of fungi differ between healthy and unhealthy gorgonians: and 3) to compare the effectiveness of different extraction methods and culture media. Ten gorgonian species from 3 families (Ellisellidae, Plexauridae, Subergorgiidae) were sampled to facilitate inter-family and inter-generic comparisons of gorgonian-fungal associations. The highest numbers of fungal isolates grew on Cornmeal agar (CMA) and Rose Bengal Agar (RBA) while CMA and Glucose Yeast Agar (GYA) gave the highest diversity of isolates. The Moist Chamber method gave the fewest numbers of isolates and the lowest diversity. All isolated fungi were Deuteromycetes. The commonest genera were sterile fungi, *Aspergillus, Penicillium,- Trichoder,na* and *Cladorporiurn,* while the *rarer* genera included *Tritirachium, G/iomastix, Sco/ecobasidium* and *Acremonium.* Fungi isolated from healthy and unhealthy gorgonians belonged to similar genera, and total numbers of isolates did not differ significantly between healthy and unhealthy gorgonians. These fungi do not appear to be causative agents of gorgonian diseases on Singapore reefs; instead, they may represent a natural flora associated with gorgonians, or saprophytes using dead tissues on unhealthy gorgonians.

Keywords Gorgonian, Fungi, Deuteromycetes, Health, Diversity

Introduction

Fungi are associated with many forms of marine organisms including sponges and algae'(Harvell et al. 1999), scleractinian corals and gorgonians, and some fungi associated with anthozoans appear to be pathogenic. Kendrick et al. (1982) isolated several bioeroding fungal species from live corals (e.g. *Acremorrium* sp., *Aspergillus* spp., *Penici/liurn* spp., *Cladosporiwn* sp). Ramos-Flores (1983) found a lower marine fungus associated with black line disease in the Caribbean *Montastrea* arum/aria, but neither its identity nor etiology has been established. Bak and Laane (1987) *observed* that high concentrations of an unknown dark fungus in the skeleton of Porter sp. caused distinct black bands; Priess el al. (2000) made similar observations, and *suggested* that the fungus may be an *Aspergillus-like* fungus. Another fungus, *Scoleco-hasidiurn* sp. was isolated from corals in Lakshadweep, India (Raghukumar and Raghukumar 1991). While all of these studies reported the presence of fungi in the skeletons of scleractinian corals, the role of these fungi remains unknown. Recently, Le Campion-Alsumard et al. (1995) began to investigate whether this association of fungi with corals is a form of symbiosis or disease.

Early reports of fungi in anthozoans were mostly from scleractinian corals, but later reports have increasingly emphasised gorgonians. Fungi are now known to cause diseases in gorgonians. Widespread disease and mortality in Caribbean gorgonians, especially *Gorgon/a* ventalina and *C. Jlabellum.* have been reported from Costa Rica and Santa Marta (Ga1z6n-Ferreria and Zea 1992), Curacoa, Saba, Trinidad, British Virgin Islands, Florida and Bahamas (Smith et al. 1996, Geiser et al. 1998), and the fungus *Aspergillus sydowif* has been shown to be responsible (Smith el al. 1996, Geiser et al. 1998). Although this is a terrestrial fungus, and is not known to complete its life cycle in marine environments, *A. sydowif* had been cultured from both coastal and oceanic zones, demonstrating that a reservoir of this organism exists in the water column (Geiser el al. 1998). Since this fungal pathogen is a well-known opportunistic -pathogen of stressed and immune-compromised hosts (Dixon and Walsh 1992), it is likely that the recent outbreaks of aspergillosis may have been triggered by environmental factors such as elevated water temperatures or increased sediment load in the water. Symptoms of infected gorgonians included recession of the coenenchyme, exposing the axial skeleton; lesions on the seafan blade that were colonized by foulers such as sponges, algae, hydroids and bryozoans (Yoshioka and Yoshioka 1991, Nagelkerken et al. 1997) and detachment and fracture of the axial skeleton (Yoshioka and Yoshioka 1991).

Despite these studies, little is known about the presence of fungi in corals. Besides *A. sydowii.* other pathogenic fungi may be present in corals without being detected (Raghukumar and Raghukumar 1991, Kendrick et at. 1992). Hence, the primary goal of this study was to describe the mycological flora associated with corals and gorgonians, as a first step towards investigating the nature of interactions between these organisms. A secondary goal was to determine the most effective way to extract and culture fungal isolates from gorgonians.

Materials and Methods

All gorgonians were collected from Raffles Lighthouse (1°09'5"N, 103°44'5"E), a fringing reef south of Singapore. Ten species were sampled from three families: Subergorgiidae *(Subergorgia suber'osa, S. moths);* Ellisellidae *(Jmmeelln* sp. A, J. *(Dichote/la)* cf *gemmacea, Ctenocella (Umbracella)* sp. A, C. cf umbraculum); Plexauridae *(Euplexaura cf pinnatn, Echinogorgia sp. A, Echinogorgia sp. C).* Samples were

[1] Department of Biological Sciences. National University of Singapore, Blk S2, Science Drive 4, Singapore 117543." email: scip9543gnus.edu.sg

collected between January and June 2000. Gorgonian colonies were found mainly on sandy bottoms at depths of 12 m to 18 m.

The study consisted of three experiments designed to: 1) establish the diversity of fungi found on gorgonians; 2) investigate differences in the fungal flora of healthy and unhealthy colonies; and 3) assess the suitability of methods used for isolating these fungi.

Experiment 1 - Surface Sterilization

An effective method for removing surface contaminants from gorgonians was established using healthy colonies from five gorgonian species. For each gorgonian species, 4 healthy colonies were collected. Each colony was cut into 16 pieces approximately 2 X 2 cm each. 4 pieces were subjected to each of the four treatments: 1) untreated; 2) washed with 0.5% sodium hypoclorite and rinsed with sterile seawater; 3) vortexed 4 times with sterile seawater; and 4) vortexed 8 times with sterile seawater. After treatment, each piece of gorgonian was plated on a sterile Glucose Yeast Agar (GYA); incubated at 27°C; and checked regularly for fungal growth. Numbers of fungal colonies were counted. Fungal colonies found growing were subcultured onto Potato Dextrose Agar (PDA) for identification; *Aspergillus* spp. and *Penicillium* spp were subcultured onto Czapek Dox Agar (CDA). Vortexing 4 times was selected as the optimal treatment and used in the other experiments.

Experiment 2 - Fungal Diversity

Four healthy and four unhealthy colonies of each gorgonian species were collected from the site. Unhealthy colonies were: 1) colonized by algae, hydroids, sponges, tunicates, bivalves or anemones; 2) had lesions or signs of predation; or 3) showed signs of discoloration. Each colony was placed in a separate sterile plastic bag and kept in ice during transport to the laboratory. All samples were processed within 24 hours of collection to prevent deterioration, and to minimize aerial contamination. Each colony was cut into 32 pieces approximately 2 X 2 cm each. The 32 pieces of gorgonian from each colony was placed in a sterile test tube with 10 ml of sterile seawater and vortexed for about 20 seconds; the water was then drained and discarded. This was repeated 3 times. Washed samples from each colony were then assigned to one of eight culture regimes: directly plated on Potato Dextrose Agar (PDA), Cornmeal Agar (CMA), Glucose Yeast Agar (GYA), Rose Bengal Agar (RBA), Malt Extract Agar (MEA), or Plain Agar; incubated in Glucose Yeast Peptone (GYP) liquid medium; incubated in moist chambers (MC). 4 replicates per colony were employed for each medium All agar media were prepared using filtered seawater with 0.2g/l of chloramphenicol to inhibit bacterial growth. GYP was prepared in a similar way but omitting agar powder. They were than autoclaved at 121°C for 15 min. The moist chambers consisted of sterile Petri dishes lined with sterile filter paper moistened with sterile seawater. After samples were placed in the Petri dishes, they were sealed with parafilm. After adding the samples, all media were incubated at room temperature (27°C) and observed daily for fungal growth. Fungi growing on the gorgonian pieces, or extending into the agar medium, were subcultured to obtain pure cultures for identification

Experiment 3 - Tissue Specificity

To determine whether types or numbers of fungi differed between axial and coenenchymal tissues of gorgonians, four healthy and four unhealthy colonies of *Junceella* cf *gemmacea* were selected. Each colony was cut into 48 pieces approximately 2 X 2 cm each and divided into 2 portions of 24 pieces each. 24 pieces of gorgonian were surface sterilized and plated onto the six agar media. For the other 24 pieces, all coenenchyme was stripped from each piece, leaving only the axis which was then surface sterilized and then plated onto the six agar media. Plates were incubated at 27°C and checked regularly for fungal growth.

Results

Experiment 1 - Surface sterilization

This experiment addressed concerns that fungi isolated from the gorgonians were actually terrestrial contaminants gained between collection and processing. The untreated samples produced approximately 33% to 48% more fungal isolates (206 total) than the treated samples (108-137) (single-factor ANOVA; $P<0.05$) (Table 1). Treatment with 0.5% sodium hypoclorite, and vortexing 4 times and 8 times with sterile seawater, consistently yielded similar numbers and types of fungal isolates, and there were no significant differences among fungi isolated from each gorgonian species are listed in Table 2.

Table 1 Numbers of fungal isolates from different washing treatments.

Gorgonian	Untreated	0.5% sodium hypochlorite	4X washing with sterile seawater	8X washing with sterile seawater
Junceella cf *gemmacea*	49	19	15	14
Ctenocella. (*Umbracella*) sp. A	44	32	43	28
Echinogorgia sp. A	29	24	14	17
Subergorgia. suberosa	40	34	27	22
Euplexaura cf *pinnata*	44	28	32	27
Total no. of fungal isolates	206	137	131	108

Experiment 2 - Fungal Diversity

There were 51 types of deuteromycetous fungi from 18 fungal taxa, 2 types of yeasts and several isolates of sterile filamentous fungi. *Penicillium* spp. from the P. *janrhlnelium* series appear to be ubiquitous to all gorgonian species tested. Sterile isolates were also isolated from all 10 species of gorgonians. A few species of fungi (such as &remind= bulge Aspergillus *kunagawcnsi,s* Nehira, *Chaetophoma* sp.) were found from 1 gorgonian species only, however the numbers of isolates of these species were too low to conclude that they were strictly associated with the particular gorgonian species. Numbers of fungi isolated ranged from 4 to 45 fungi in healthy gorgonians, and from 19 to 39 in unhealthy gorgonians (Table 2). Overall, unhealthy gorgonians yielded more fungal isolates (291) than from healthy gorgonians (231). Of the 10 gorgonian species tested. only *Junceella sp.* A, *J.* cf gernmacea, *Ctenocela* cf *tonbracuhan* and *Echinogorgia* sp. C had significantly more fungi *on* unhealthy colonies (Table 2) (single-factor ANOVA, $P<0.05$),

Comparisons of the various culture regimes and media show that the moist chamber incubation yielded almost no fungi (Table 3). The liquid medium method (GYP) also *gave* very few fungal isolates: about 22% fewer taxa and about 53% fewer isolates than most agar media. Among the agar media, Plain Agar had the same number of taxa as GYP, but nearly as many isolates as other agars. The five enriched agars (CMA, PDA, (WA, MEA, RBA) were equally suitable for culturing a similar diversity of fungi, and similar numbers of isolates. Fungi from P. *cirrinum* series, *P. janthinellum* series and Black yeast were isolated from all media used except for Moist Chamber method. White yeast and C. *sphaerosperrnum* were isolated from all media except GYP. *Fu.sarium sp.* was only isolated from GYA and GYP. The rest of the fungal isolates were isolated in very small numbers and cannot be concluded to be media-specific.

Experiment 3 - Tissue Specificity

Significantly more fungal types and isolates were obtained from the axes + coenenchyme complex of J. cf *gernrnacen* than from the axes alone (Table 4). In healthy colonies, only 3 isolates from 2 taxa grew from the axes, while the axes + coenenchyme complex yielded 47 fungal isolates from 8 taxa. The axes from unhealthy colonies yielded more fungal isolates than axes of healthy colonies. The axes + coenenchyme complex of unhealthy colonies had less than half the number of isolates (22) compared to that of healthy colonies (47 isolates). Unlike the healthy gorgonians, unhealthy colonies produced similar numbers of isolates from both axes and axis + coenechyme complexes (single-factor ANOVA, $P<0.05$). It was observed that *Cladosporium splmerosperrnum* and White yeast was isolated from the axis+coenenchyme complex only and not from the axis alone.

Discussion

Experiment I showed that the diverse range of fungi associated with gorgonians was not a result of surface contamination. Untreated samples had the highest diversity and abundance of fungi, while 0.5% sodium hypochlorite, which is commonly used to surface-sterilize scleractinian corals, reduced the numbers by approximately 33%. Due to-the fact that gorgonian tissues are much thinner than scleractinian tissues,' and prolonged treatment with sodium hypochlorite might have killed some fungi growing inside the tissue; we decided that vortexing with sterile seawater was a better method for surface sterilization. Agitation of samples at high speed should dislodge fungal mycelia and spores that are attached superficially. As vortexing the samples with sterile seawater did not significantly reduce the incidence of fungi, compared to the sodium hypochlorite treatment. and since increasing the number of times of vortexing also did not reduce fungal numbers, we conclude that the majority of fungi isolated were true associates of gorgonians and not accidental surface contaminants. The extra isolates from the untreated samples were of the same taxa as the treated samples. These isolates *were* probably just loosely attached to the gorgonians or may be terrestrial contaminants. However, we were looking for true associates of gorgonians which must be- growing within the gorgonian tissues.

Many concepts of biodiversity include both species richness (number of taxa) and their relative abundances (i.e. number of isolates). While overall, there were significantly more fungal types isolated from unhealthy colonies than healthy colonies, this phenomenon was true for only four of the gorgonian species: *Junceella* sp. A, *Junceella cf genunacea,*. *Clenocella* cf *umbra'ulum* and *Echinogorgia* sp. C. The other six gorgonian species did not show this trend. Moreover, the species of fungi obtained were similar for both healthy and. unhealthy colonies of all gorgonian species.

The results provide no evidence to suggest that fungi were acting as causal agents on unhealthy gorgonians in Singapore. This differs from the Caribbean where the terrestrial *Aspergillus sydowii* has been identified as the pathogen causing a widespread disease of gorgonians (Smith et al. 1996; Geiser et al. 1998). *A. sydowii* was not among the Aspergillus spp. isolated from gorgonians in Singapore although it does exist on land in S. E. Asia (Thom and Raper 1945). The absence of pathdgcnicity could mean that diseases in Singapore lack discrete symptoms, or that they were not present at the time of sampling.

. The presence of diverse fungal assemblages on healthy gorgonian colonies (even after surface sterilization -),. suggests that fungal communities' are naturally associated with gorgonians. The higher number of fungi found on certain species of unhealthy gorgonians could be attributed to unhealthy gorgonians having lower resistance against invasion by foreign organisms, or the dead tissues on unhealthy gorgonians may encourage growth of saprophytic fungi.

Table 2 Fungi isolated from healthy and unhealthy colonies of 10 gorgonian species

Fungal Taxa	Subergorgiidae						Ellisellidae								Plexauridae					
	1		2		3*		4*		5*		6		7		8		9		10	
	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U
Acremonium butryi										1										
A. furcatum																		1		1
A. strictum						1			1										1	
A. aculeatus		2			1															
A. cervinus Massee							1		1		1									
A. foetidus var. *pallidus*						1				1										
A. kangawensis Nehira		1																		
A. nutans													1	1						
A. pulverulentus	2																			
A. ficuum		2		2												1				
A. flavus Link												2							1	
A. orchraceus series											3									
A. ornatus series						1					6	3	1	1			1		2	1
A. terricola Marchal											4	2								
A. terricola var. *indicus*											1									
A. wentii																		1		
Chaetophoma sp.																		1		
Cladosporium musae	5															1			1	1
C. sphaerospermum	1			1		1		10	4		1	4		1			4	3	3	2
Fusarium sp.	2	1																		1
Gliomastix cerealis													1			1				
G. luzulae																1				
G. murorum var. *felina*		1	1																	
Hymenula sp.		1																		
Microascus triganosporus																			1	
Oidodendron griseum						1														
Penicillium brevi-compactum																				1
P. camemberti series				1															2	
P. canescens series																			1	
P. citrinum series	2	5						1		2		1				1		1	1	2
P. decumbens Thom							1													
P. frequentans series	2	1														1	1			1
P. implicatum series		1	9	1			1				1	1	2					2		1
P. janthinellum series	4	2		1		1		3	1	2	5	3	1			1	1		2	3
P. lanoso viride Thom											1									
P. lilacinum Thom	3						3									1				
P. notatum Westling											1									
P.oxalicum Currie & Thom	1																			
P. steckii Zaleski														1						
Phoma-like						1								1						
Scolecobasidium humicola	2		1	1	1	2	1		1	1			1	1				1	2	
Sporotrichum sp.		1																		
Stibella sp.													1							
Trichoderma pseudokoningii	1			1									1			1				1
T. hamatum															1					
T. harzianum	1	1											1						1	
T. koningii	1																			
T. longibrachiatum	1	1																	1	
Tritirachium sp.	2												2					1		
Verticillium sp.														4					2	
Virgaria sp.								1												
Black yeast			3	3		2	4		1	1	3	1		1	4			2	1	
White yeast				1		1	1			2		3						2	3	
Sterile isolates	15	19	17	20	2	7	5	14	2	14	2	10	17	18	17	26	9	10	2	14
No. of fungal isolates	45	39	31	32	4	19	17	29	11	24	29	30	29	29	22	35	16	25	27	29
Total no. of fungal taxa	23	11	14	12	11	15	16	12	14	23										

Key: Gorgonians: 1) *Subergorgia suberosa*, 2) *S. mollis*, 3) *Junceella* sp. A, 4) *J.* cf *gemmacea*, 5) *Ctenocella* cf *umbraculum*, 6) *C. Umbracella* sp. A, 7) *Echinogorgia* sp. A, 8) *E.* sp. C, 9) *E.* sp. E, 10) *Euplexaura* cf *pinnata*. The numbers in the table denotes the number of isolates for each fungal taxon. This is based on 4 replicates for each gorgonian species. (*) denotes significant differences in the number of fungal isolates between healthy and unhealthy colonies.

Table 3 Numbers of fungal taxa and fungal isolates obtained from gorgonians using eight differerent isolation methods and culture media

	CMA	GYA	MEA	PDA	Plain	RBA	GYP	MC	Total
No. of fungal taxa	23	24	18	22	18	20	18	2	51
No. of isolates	84	78	82	81	71	91	38	2	522

Key: CMA: Cornmeal Agar; GYA: Glucose Yeast Agar; MEA: Malt Extract Agar; PDA: Potato Dextrose Agar; Plain: Plain Agar; RBA: Rose Bengal Agar; GYP: Glucose Yeast Peptone (liquid medium); MC: moist Chamber

Table 4 Types and number of fungal isolates obtained from the axis and axis+coenenchyme complex of *Junceella* cf *gemmacea*

Fungal Taxa	Gorgonians with axis only		Gorgonians with axis+coencenchyme	
	Healthy	Unhealthy	Healthy	Unhealthy
Aspergillus spp.	1		1	1
Cladosporium sphaerospermum			1	1
Monosporium sp.				1
Penicillium spp.		1	3	
Scolecobasidium humicola			1	
Trichoderma spp.		1		1
Verticillium sp.				1
Black Yeast		1	16	1
White Yeast			7	1
Sterile isolates	2	16	18	15
Subtotal	3	19	47	22
Total no. of fungal isolates		22		69

The data showed clearly that incubating samples in moist chambers, liquid medium, and low nutrient media such as plain agar, are inadequate methods for detecting fungi associated with gorgonians. However, all high nutrient agars (PDA, CMA, MEA, GYA, RBA) yielded similarly high numbers and types of fungi. We also conclude, from inspection of the specificities of the fungi for particular media, that a combination of only two media (GYA and PDA) would be sufficient for isolating fungi from gorgonians.

In both healthy and unhealthy gorgonians, higher number and more types of fungi were extracted from the axis + coenenchyme complex than from the axis alone, however this is not significant for unhealthy gorgonians. While unhealthy axes yielded more fungi than healthy axes, the unhealthy axes + coenenchyme complex yielded fewer fungi than the healthy axes + coenenchyme complex. This suggests that fungi in healthy gorgonians mainly inhabit the outer coenenchyme surface and rarely penetrated deep enough to reach the axes. The results also suggest that gorgonian coenenchyme is a good substrate for fungal growth. This may be because unhealthy colonies had lost most of their outer tissues, leaving much less coenenchyme to habour fungi. In addition, if the coenenchyme is no longer intact, fungi may be able to penetrate more easily to the axes.

This study gave a better picture of the types of fungi associated with gorgonians. The fact that a diverse array of fungi was associated with gorgonians suggested that they could play important roles in the ecosystem. Further studies done on this aspect may be able to provide answers to the sources of these fungi, their disease-causing implications as well as their route of infection and spread.

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