Fungi in *Porites lutea*: association with healthy and diseased corals

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ABSTRACT: Healthy and diseased scleractinian corals have been reported to harbour fungi. However, the species of fungi occurring in them and their prevalence in terms of biomass have not been determined and their role in coral diseases is not clear. We have found fungi to occur regularly in healthy, partially dead, bleached and pink-line syndrome (PLS)-affected scleractinian coral, *Porites lutea*, in the reefs of Lakshadweep Islands in the Arabian Sea. Mostly terrestrial species of fungi were isolated in culture from these corals. Hyaline and dark, non-sporulating fungi were the most dominant forms. Fungal hyphae extended up to 3 cm within the corals. Immunofluorescence detection using polyclonal immunological probes for a dark, initially non-sporulating isolate (isolate # 98-N28) and for a hyaline, non-sporulating fungus (isolate # 98-N18) revealed high frequencies of these in PLS-affected, dead and healthy colonies of *P. lutea*. Total fungal biomass accounted for 0.04 to 0.05 % of the weight of corals in bleached corals and was higher than in PLS-affected and healthy colonies. Scanning electron microscopy revealed the presence of fungi within the carbonate skeleton and around polyps. Fungi appear to be a regular component of healthy, partially dead and diseased coral skeleton.

KEY WORDS: Scleractinian coral · *Porites lutea* · Bleaching · Pink-line syndrome · Fungi · Immuno-fluorescence probe · Lakshadweep Islands · Arabian sea · India

INTRODUCTION

Several publications document the occurrence of fungi in healthy and diseased corals (Ramos-Flores 1983, Le Campion-Alsumard et al. 1995a, Smith et al. 1996). Kendrick et al. (1982) isolated terrestrial species of fungi in culture from a number of species of live corals and Le Campion-Alsumard et al. (1995a) suggested a symbiotic role for fungi in healthy corals. Raghukumar & Raghukumar (1991) found them in necrotic patches of a massive coral. A number of new fungi have been described from dead corals (Kohlmeyer & Volkmann-Kohlmeyer 1987).

Because of widespread mortality of coral reefs, documenting coral diseases has assumed importance in recent years and the spread of several old and new diseases has been reported from various parts of the world (Kushmaro et al. 1996, Goreau et al. 1997, 1998, Richardson 1998). Pathogenic organisms are identified for some of these, whereas for others the causal organisms are not yet known. Fungi are well known as plant and animal pathogens on land, and several fungal diseases have also been reported from the marine environment (Raghukumar 1996, Richardson 1998). A terrestrial species of fungus has also been shown to cause a disease in soft corals (Smith et al. 1996). In view of these reports, it is of interest to examine whether fungi associated with diseased corals are primary pathogens. We investigated the presence of fungi, and their species composition, prevalence and biomass in healthy, partially dead, bleached and pink-line syndrome (PLS)-affected scleractinian coral *Porites lutea*.

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MATERIALS AND METHODS

Study area. Diseases of corals were surveyed in the lagoon and intertidal zones of Kavaratti Island of the Lakshadweep archipelago off the south-west coast of India. The Lakshadweep archipelago comprises 36 islands with 12 atolls and lies between 10–12° N and 71° 40′–74° E in the Arabian Sea (Fig. 1) and forms the northern part of the Laccadive-Chagos Ridge. Samples of *Porites lutea* were collected in the lagoon at a depth of 3 to 5 m and samples of *P. compressa* were collected from the intertidal zone of reef flat in Kavaratti Island. Bleached and PLS-affected corals were collected in October and November 1998 and April 1999.

Frequency of fungi isolated from corals. Small pieces from the surface of healthy-looking colonies of *Porites lutea* and those showing PLS were chiselled and collected in sterile plastic bags under water. The samples were taken in ice and processed within 2 h of collection. The coral samples were broken further into smaller pieces inside the same plastic bags, thereby avoiding exposure of the broken surface to air to avoid contamination by aerial opportunistic fungi. The broken pieces ranging in size from 3 to 5 mm^3 were directly plated on 1:5 diluted corn meal agar medium (HI-MEDIA, Mumbai, India) prepared with half-strength sea water and fortified with streptomycin (0.5 g l^{-1} medium) and penicillin (100 000 U l^{-1} medium) to inhibit bacterial growth. The presence of aerial fungi in the experimental site was tested by exposing 3 to 5 Petri plates containing corn meal agar medium to the air for 10 s during the isolation procedure. Subsequent comparison of these plates with experimental plates facilitated the detection of aerial contamination in isolation plates. Fungal hyphae emerging from coral pieces after 5 to 7 d of incubation in the dark were identified up to generic level. Frequency of fungi isolated from corals is expressed as a percentage.

Estimation of biomass of fungi. Coral samples from the polyp zone (up to 5 mm below surface) were weighed and decalcified in a decalcification solution (1:1 ratio of 20% citric acid 50% formic acid). The resulting material was homogenised in a VirTis homogeniser (VirTis Co., Gardiner, NY, USA) at 5000 rpm for 15 s and filtered over a 0.22 μm pore size Nuclepore polycarbonate filter paper under vacuum. The filter paper was stained with 0.01% Calcofluor, an optical brightener that stains chitin and cellulose (Sigma, St Louis, MO, USA) for 5 to 10 s (Mueller & Sengbusch 1983). After the excess stain was washed with distilled water, the filter was mounted with a drop of non-fluorescent immersion oil and a cover slip was applied. The slides were stored in the dark at 5°C until further examination.

The slides were observed under an epifluorescence microscope with a blue excitation filter (Olympus BX-60, Tokyo, Japan). The images of fungal hyphae were captured with a charge-coupled device camera (Optronics Engineering, Goleta, CA, USA) and digitised using a frame-grabber card (Oculus TCX, Coreco Inc., Quebec, Canada). Since some coral detritus also showed fluorescence, images were manually traced in conjunction with Optimas 6.0 image analysis software (Optimas Corporation, Bothell, WA, USA) calibrated to the particular objective. Total length and average width of the mycelia for the total area were estimated using the software to calculate fungal biovolume. Mycelial fungal biomass was determined by assuming a mass density of 0.2 g cm^{-3} (Newell et al. 1986) and expressed as mg fungal biomass g^{-1} wet weight of
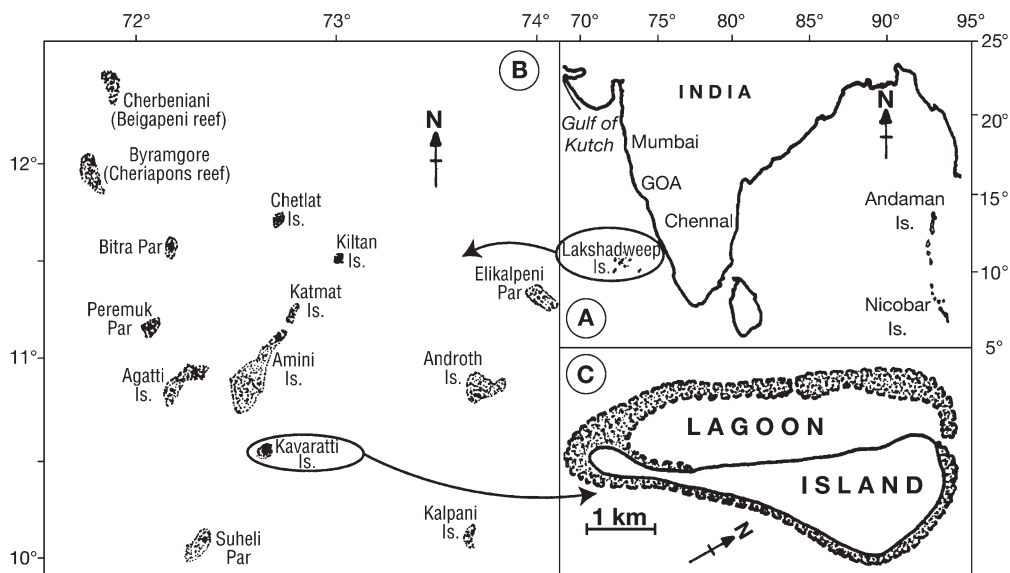

Fig. 1. Site of sampling: (A) Lakshadweep islands situated on the south-west of India in the Arabian Sea; (B) the group of islands of the Lakshadweep archipelago; (C) Kavaratti island with lagoon and a reef enclosing lagoon and island

coral tissue. About 3 to 10 coral pieces were examined as replicates for this study.

Vertical distribution of fungi in corals. PLS-affected coral, the dead portion from partially dead corals and healthy-looking colonies of *Porites lutea* were cut into 1 cm thick sections with an electric rock cutting saw. These sections were washed with a jet of water to remove free particles and stained with 0.01% Calcofluor solution for 2 to 3 min. After excess stain was washed off, the sections were examined under an epifluorescence microscope, using a blue excitation filter for the presence of fungi. Dark, non-sporulating (DNS) fungi did not stain with Calcofluor but could nonetheless be detected directly. The results are qualitatively expressed as presence or absence of fungi at various depths in the coral sections.

Scanning electron microscopy of corals. Healthy-looking pieces of *Porites lutea* and those showing PLS collected from a 1 to 3 m depth in the lagoon were fixed immediately with 3% formalin prepared with sea water for 24 h. They were further fixed with a 10% formalin solution for 48 h. The fixed samples were rinsed thrice with tap water and were preserved in 70% ethanol and stored till use. Fixed samples were later decalcified (see above) and washed twice in distilled water and dehydrated in a graded acetone series. The tissues were dried critically using liquid CO_2 in a critical point freeze drying apparatus (Structure Probe Inc., West Chester, PA, USA). The whole specimens (containing tissue and skeleton) were prepared in a similar manner without the decalcification step for scanning electron microscopic (SEM) studies. Critically dried specimens were sputter coated with gold-palladium and examined under an SEM to observe the fungi (model 5800 LV, JEOL, Akishima, Japan).

Immunofluorescence detection of selected fungi in *Porites lutea*. Antibodies were raised commercially (Genei India Pvt. Ltd, Bangalore, India) for 2 of the most frequently isolated fungi, a dark mycelial fungus (isolate # 98 N-28, *Curvularia lunata* (Wakker) Boedijn) and a hyaline, non-sporulating (HNS), unidentified isolate (Isolate # 98 N-18) obtained from a partially dead and a PLS-affected coral, respectively. About 2 mg of fungal pellet was crushed in 2 ml 0.15 M NaCl and centrifuged, and 1 ml supernatant was emulsified with 1 ml Freund's adjuvant. This was injected subcutaneously at multiple sites on the back of New Zealand male white rabbits (2 rabbits for each fungus). The first booster injection was given after 6 wk of the primary injection and the second booster was given after a further 4 wk. The antibody titre was monitored by Dot ELISA after 10 d of the booster injection and yielded a titre value of 1:2000 for isolate # 98 N-18 and 1:5000 for isolate # 98 N-28. The antibodies thus obtained were diluted 4 times in phosphate buffered saline (PBS) con-
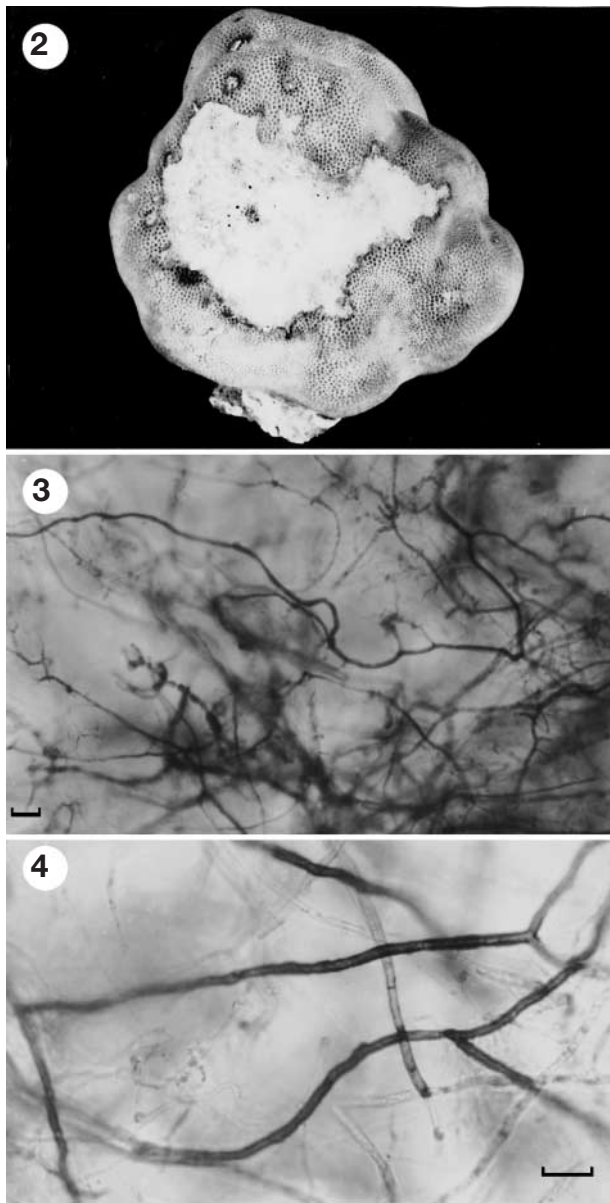
taining 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4 in 1000 ml distilled water (Mendoza et al. 1995). The coral specimens were crushed into a coarse powder and incubated with 50 µl of the diluted antibody solution in an Eppendorf tube for 1 h at 25°C. The mixture was vortexed after dilution to 1000 µl with PBS solution. The slurry was centrifuged at 4000 rpm for 1 min. After the supernatant was decanted, the coral debris was similarly washed 3 times with 1000 µl of PBS. The samples were incubated in 50 µl of the secondary antibody (Genei India Pvt. Ltd), and the goat anti-rabbit anti-serum was tagged with fluroscein isothiocyanate for 1 h at 25°C. The excess stain was washed with PBS as described. The coral debris was transferred onto a slide and viewed under blue excitation (excitation wave length 400 to 440 nm and barrier filter # 475) for the presence of fungi and photographed. Specificity of the antiserum was confirmed by the absence of immunofluorescence staining by 4 species of *Aspergillus*, *Cladosporium* sp. and mycelial yeast isolated from *P. lutea*. Staining of coral sections with preimmune sera did not show any immunofluorescence.

Histology. To detect fungi within polyps, coral polyps were removed from formalin fixed coral pieces following decalcification as described above. The polyps were dehydrated in a graded series of ethanol and embedded in wax and sectioned. The sections were stained with basic fuchsin (Bancroft 1995).

RESULTS

Bleached corals were collected 5 mo after the major coral reef bleaching event reported from all over the world including the Andamans in the Bay of Bengal (Ravindran et al. 1999). Line transect observations carried out in October 1998 and April 1999 in the lagoon at Kavaratti Island revealed the presence of bleaching and PLS in *Porites lutea* colonies. In the PLS-affected corals, the coral tissue around the dead patch appeared bright pink in otherwise pale brown morphotypes of *P. lutea* (Fig. 2).

Several fungi from PLS-affected corals as well as from dead, bleached and healthy ones were isolated from the surface of the coral up to 5 mm depth (Table 1). The highest frequency of fungi occurred in dead patches. There were no differences in the frequencies of fungi occurring in healthy and PLS-affected colonies of *Porites lutea*. Most of the identified species of fungi belonged to terrestrial genera. DNS and HNS forms were the most dominant in all the healthy-looking and stressed corals (Table 1). One of the DNS strains, # 98 N-28, isolated from a dead patch during 1998 sporulated in subsequent culture, although poorly, and was identified as *Curvularia lunata* (Wakker) Boedijn. The

Figs 2 to 4. Pink-line syndrome in a *Porites lutea* colony in the lagoon of Kavaratti. Fig. 3. Fungal hyphae and algal filaments from the polyp zone, visible after decalcification of the surface layer. Scale bar = 20 µm. Fig. 4. Septate hyphae of a dark, non-sporulating fungus seen after decalcification of the surface layer. Scale bar = 20 µm

intertidal zone. Here too the HNS (about 30% frequency) and DNS fungi (about 60% frequency) were isolated.

Sections of corals stained with Calcofluor revealed the presence of fungi as bright blue septate filaments under the epifluorescence microscope in contrast to bright orange cyanobacteria or red chlorophycean algal filaments. The highest fungal biomass occurred in bleached corals collected in October and November 1998, nearly 5 mo after the major bleaching event, whereas the fungal biomass was lower in corals with dead necrotic patches and very negligible in PLS-affected corals (Fig. 5). Examination of the depth-wise distribution of the hyaline and dematiaceous fungi revealed that they pervaded the PLS-affected corals and necrotised patches of partially dead corals more frequently than the healthy ones (Fig. 6).

An isolate of HNS fungi (# 98 N-18) as well as *Curvularia lunata* (# 98 N-28) were detected in corals by the immunofluorescence probes (Figs 7 & 8). Branched mycelia of both the fungi adhered closely to calcium carbonate particles and often appeared to be present within the carbonate skeleton (Figs 7 & 8). Frequencies of *Curvularia lunata* (isolate # 98 N-28) were similar in healthy, PLS-affected and dead corals (about 50% frequency). The HNS form (isolate # 98 N-18) was found to be more frequent in the healthy (60% frequency) and partially dead corals (80% frequency) than in PLS-affected corals with 50% frequency (Fig. 9).

SEM of the healthy, bleached and PLS-affected corals showed that fungi perforated the coral skeleton (Figs 10, 11 & 12). The hyphae showed distinct swellings at regular intervals (Figs 10 & 11). Fungal hyphae showed basal swellings on emergence from the carbonate skeleton (Figs 12 & 13). Occasionally, we also observed 'conidia-like' structures within the coral skeleton (Fig. 14). Ramifying fungal hyphae emerged

other dominant and frequently isolated fungi from these corals were various species of *Aspergillus*, a species of *Cladosporium* and a mycelial yeast. On decalcification of the top 5 mm layer, black septate fungal mycelia interspersed with thin hyaline fungal mycelia, and cyanobacterial and algal filaments were observed (Figs 3 & 4).

Fungi were isolated from almost all healthy as well as PLS-affected colonies of *Porites compressa* from the

Fig. 5. Fungal biomass in mg g^{-1} coral wet weight. Preweighed crushed corals were stained with the optical brightener Calcofluor stain and biovolume of fungi present was calculated using the Image analysis software. The fungal biovolume was converted to fungal biomass as described under 'Materials and methods'

Table 1. Fungi isolated from the polyp zone of *Porites lutea* collected from the lagoon in Kavaratti, Lakshadweep Island. Dead patch: coral with necrotic patch; ND: no data; PLS: coral showing pink-line syndrome

Fungal species	Frequency of isolation (%)					
	Healthy		PLS		Dead patch	
	1998	1999	1998	1999	1998	1999
Dark, non-sporulating forms	22	0	0	25	60	ND
Orange, non-sporulating forms	6	0	0	0	0	ND
Hyaline, non-sporulating forms	0	36	25	0	7	ND
Acremonium sp.	0	0	0	0	13	ND
Fusarium sp.	11	0	0	0	0	ND
Aspergillus sp.	17	7	25	15	7	ND
Cladosporium sp.	0	7	8	5	0	ND
Mycelial yeasts	0	21	0	0	0	ND
Labyrinthula sp.	0	0	0	5	0	ND
Unidentified	0	0	0	5	0	ND
Chaetomium sp.	0	0	0	10	0	ND
Aureobasidium sp.	0	0	0	5	0	ND
Total	56	71	58	70	87	ND

DISCUSSION

The scleractinian coral *Porites lutea* in 4 different stages of health were examined in this study. The first of these is corals affected by the pink line, a syndrome being reported for the first time (PLS). A similar syndrome was reported to occur in massive corals from Sri Lanka in 1998 (P. Weerakkody pers. comm.). It is possible that lowered host resistance due to stress-related factors induced the PLS. We are investigating the biochemical nature of the pink band observed in these corals. The pink band between the dead and healthy coral tissues may play a role in the coral's immunological and chemical defence systems similar to the pink band observed in the tissues of

from the coral skeleton and spread around the polyps (Figs 15, 16 & 17). The fungal hyphae were covered by remineralised carbonate precipitate (Figs 10, 13 & 17) and most often fresh accretion of amorphous repair carbonate around fungal hyphae was noticed (Figs 12 & 13). Whole polyps removed after careful decalcification of the surface layer of coral in the pink-line zone also revealed the presence of fungal hyphae around the periphery of polyps (Fig. 18). Sections of polyps from healthy and PLS-affected corals stained with basic fuchsin showed abundant fungal hyphae around the polyps (Fig. 19).
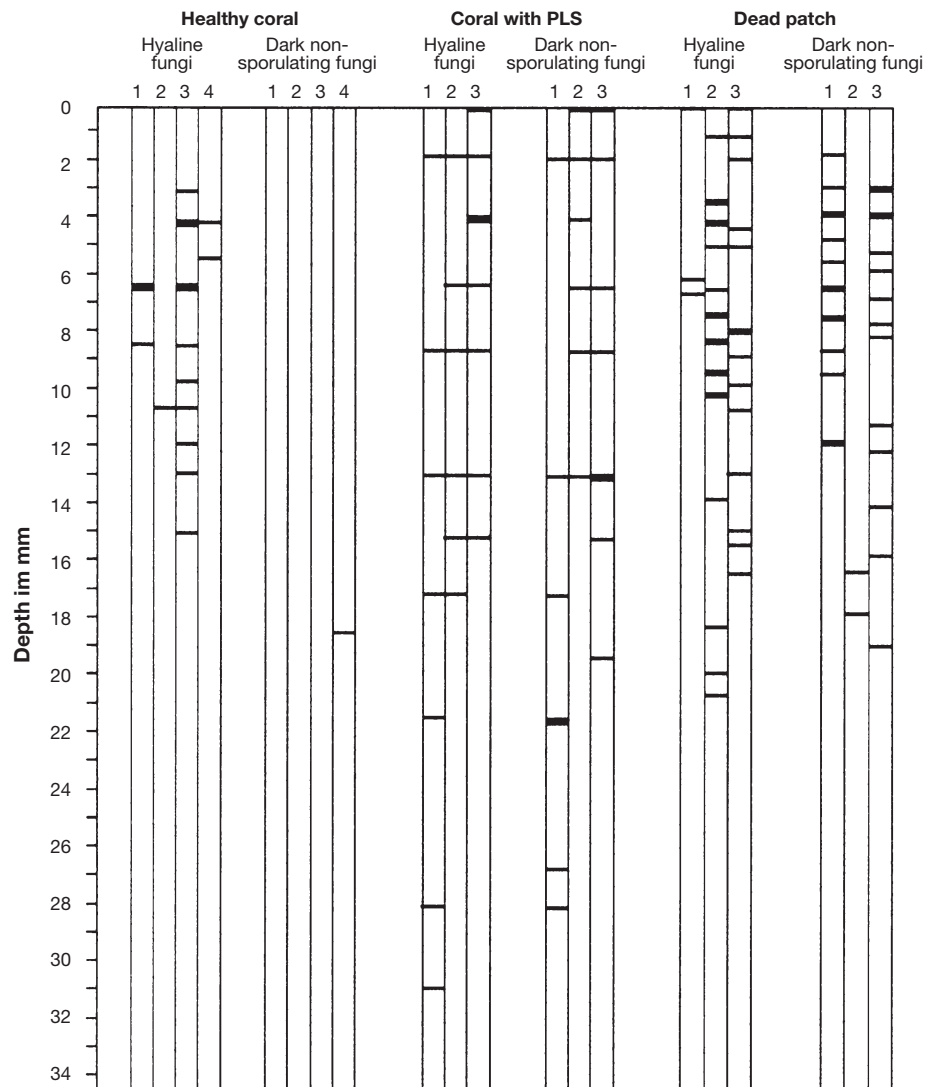

Fig. 6. Vertical sections of coral. Vertical distribution of fungi within healthy, pink-line syndrom-affected and partially dead corals. The horizontal bars represent fungal presence detected by Calcofluor staining (as described in 'Materials and methods') at a particular depth in coral. The dark bars represent detection of fungi in more than 2 transects in a vertical section of the coral. Numbers 1 to 4 represent replicate coral colonies examined
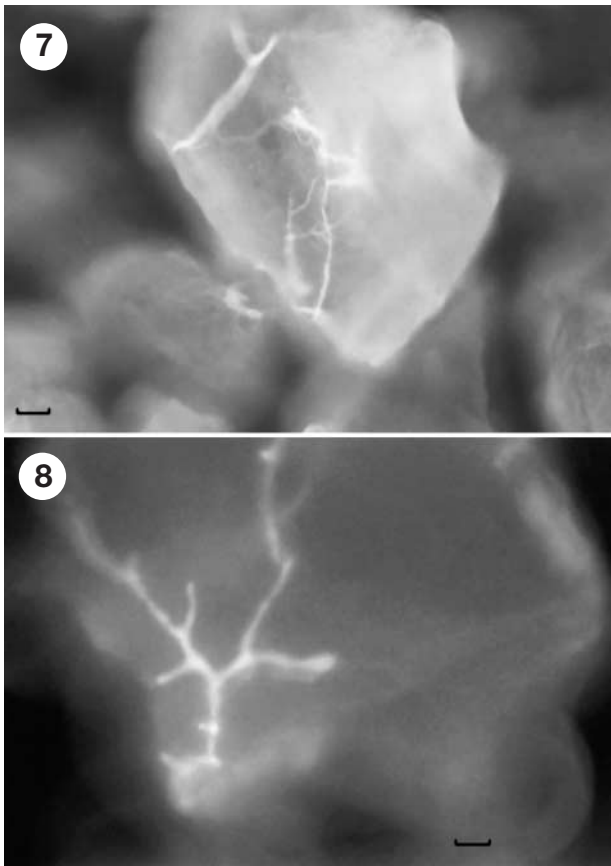
Fig. 7. Immunofluorescence detection of the hyaline, non-sporulating fungus strain 98 N-18 inside coral. Scale bar = 20 μm. Fig. 8. Immunofluorescence detection of the dark, non-sporulating fungus strain 98 N-28, *Curvularia lunata*, inside coral. Scale bar = 10 μm
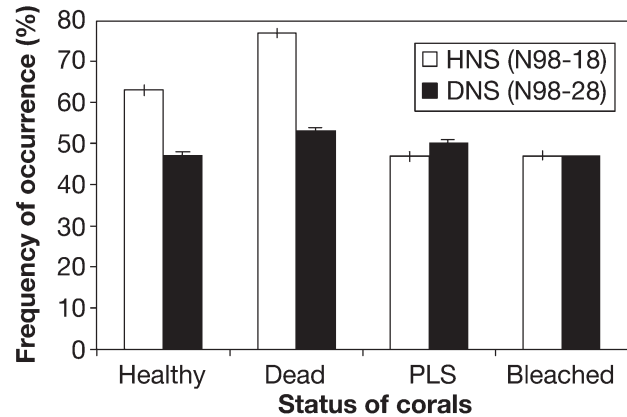

Fig. 9. Percentage frequency occurrence of the hyaline, non-sporulating (HNS) (strain 98 N-18) and dark, non-sporulating (DNS) forms (*Curvularia lunata*, strain 98 N-28) in healthy, dead and PLS-affected corals of *Porites lutea*, detected by immunofluorescence probes

pink colonies of *Pocillopora damicornis* at the contact sites between non-similar colonies (Dove et al. 1995). We also studied 'bleached' specimens of *P. lutea*. A coral-reef bleaching event is 1 of the examples of a response to environmental stress such as elevated temperature, salinity or illumination, excessive sedimentation, turbidity or chemical pollution (Williams & Williams 1990). In addition to the above, we examined healthy-looking corals devoid of any symptoms of damage and disease, as well as necrotised and dead patches on 'partially dead corals' (Meesters et al. 1996).

Our studies show that fungi are a regular component of corals. The present study was carried out over a period of 2 yr and fungi were seen to be constantly associated with healthy-looking, partially dead, bleached and PLS-affected corals. They were found extending to substantial depths within corals. Data presented show their distribution in corals up to a depth of only 3 cm but they were observed occasion-

ally at depths of nearly 7 to 8 cm and sometimes even deeper. No regular pattern of fungal distribution within corals was observed similar to the black banding seen in *Porites lutea* from Indonesian reefs (Bak & Laane 1987). Black bands in sections of *P. lutea* and *P. lobata* skeletons in Mayotte Island in the Mozambique Channel and Moorea Island in French Polynesia were attributed to the secretion of organic dark pigments by fungi and their conidia (Priess et al. 2000). In our study, we observed a highly patchy distribution of fungal mycelia within the corals. This resulted in a high variability between replicates used for calculating the total biomass, culturable numbers and frequencies as observed by immunofluorescence. Fungal biomass constituted up to 0.05 % of the weight of dead corals. This value is even higher than that reported by Raghukumar et al. (1995) for submerged detritus of leaves of the mangrove *Rhizophora apiculata* Blume (0.015 % of the detrital weight). Fungal biomass of 3 to 5 mg cm^{-3} has been reported in necrotic patches of corals from the Andaman Islands in the Bay of Bengal on the east coast of India (Raghukumar & Raghukumar 1991). Fungi, therefore, are an apparently important component of corals.

Little is known of the diversity of fungi that inhabit corals. Certain 'obligate marine fungi' have been described from dead coral slabs (Kohlmeyer & Volkmann-Kohlmeyer 1987) and the term 'facultative marine fungi' has been coined to accommodate the possibility of terrestrial species of fungi having adapted to the marine environment. Raghukumar & Raghukumar (1998, 1999) hypothesised that terrestrial fungi are indeed active in the sea. Kendrick et al. (1982) have isolated species of *Aspergillus*, *Cladosporium* and *Acremonium* in culture from healthy *Porites* colonies in the Caribbean and the
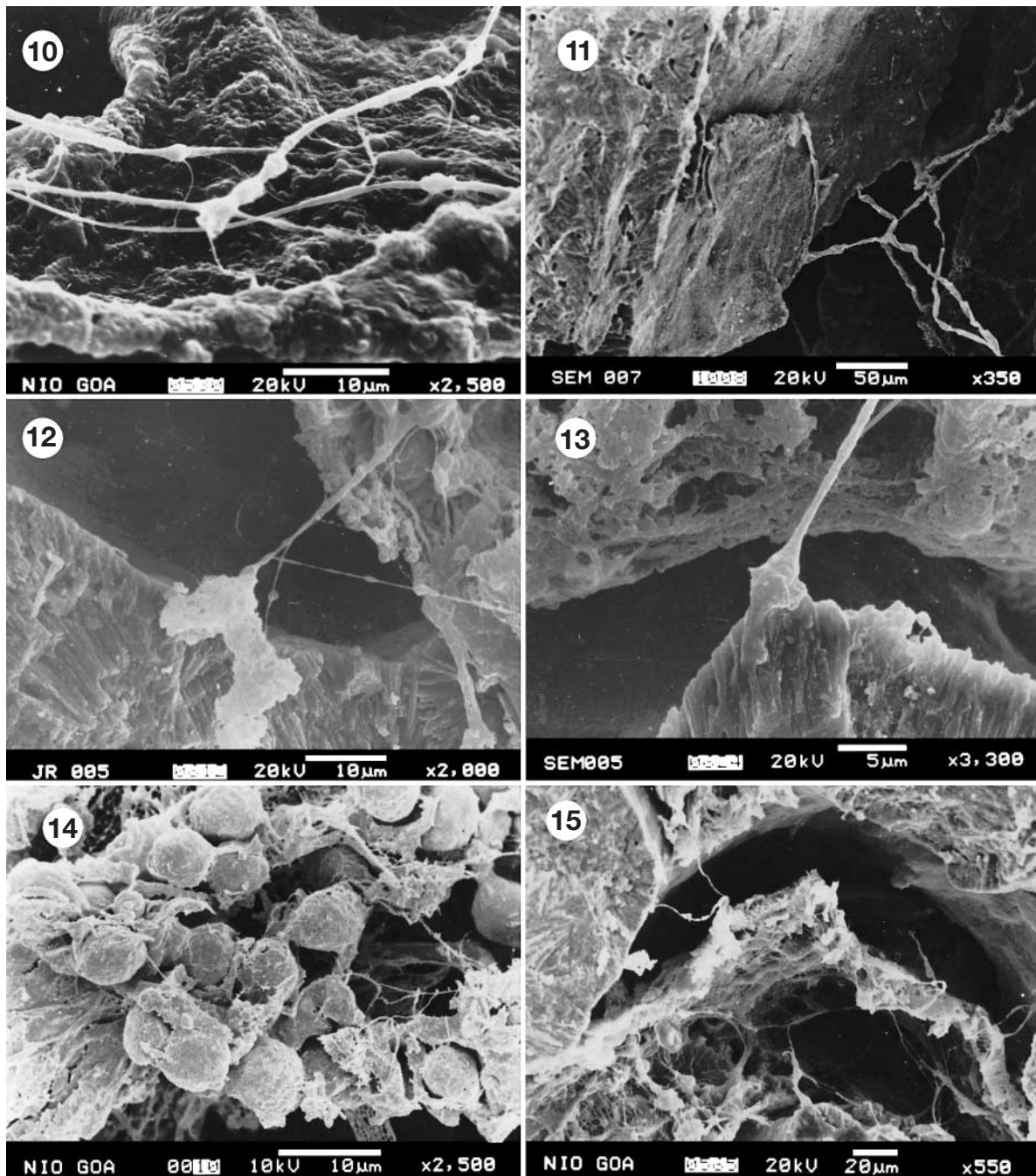
Figs 10 & 11. Fungal hyphae with swellings ramifying within coral skeleton as observed under a scanning electron microscope. Fig. 12. Deposition of amorphous repair carbonate around the invading fungal hypha. Fig. 13. A fungal hypha showing basal swelling at the site of emergence from the carbonate skeleton. Fig. 14. A group of 'conidia-like' structures. Fig 15. Fungal hyphae travelling between polyp and carbonate skeleton

South Pacific. Fungi have been isolated from bleached corals of *Millipora complanta* (Te Strake et al. 1988). In the present study, we consistently cultured terrestrial fungi from corals under different health conditions. In addition, we have established the presence of the common terrestrial fungus, *Curvularia lunata* (# 98 N-28), within healthy and PLS-affected corals using polyclonal antibodies. This fungus was labelled as a DNS form in our initial isolations. We isolated several other DNS forms in our study, although their identity is not known. However, it is possible that these too were terrestrial fungi, possibly belonging to *Curvularia lunata*.
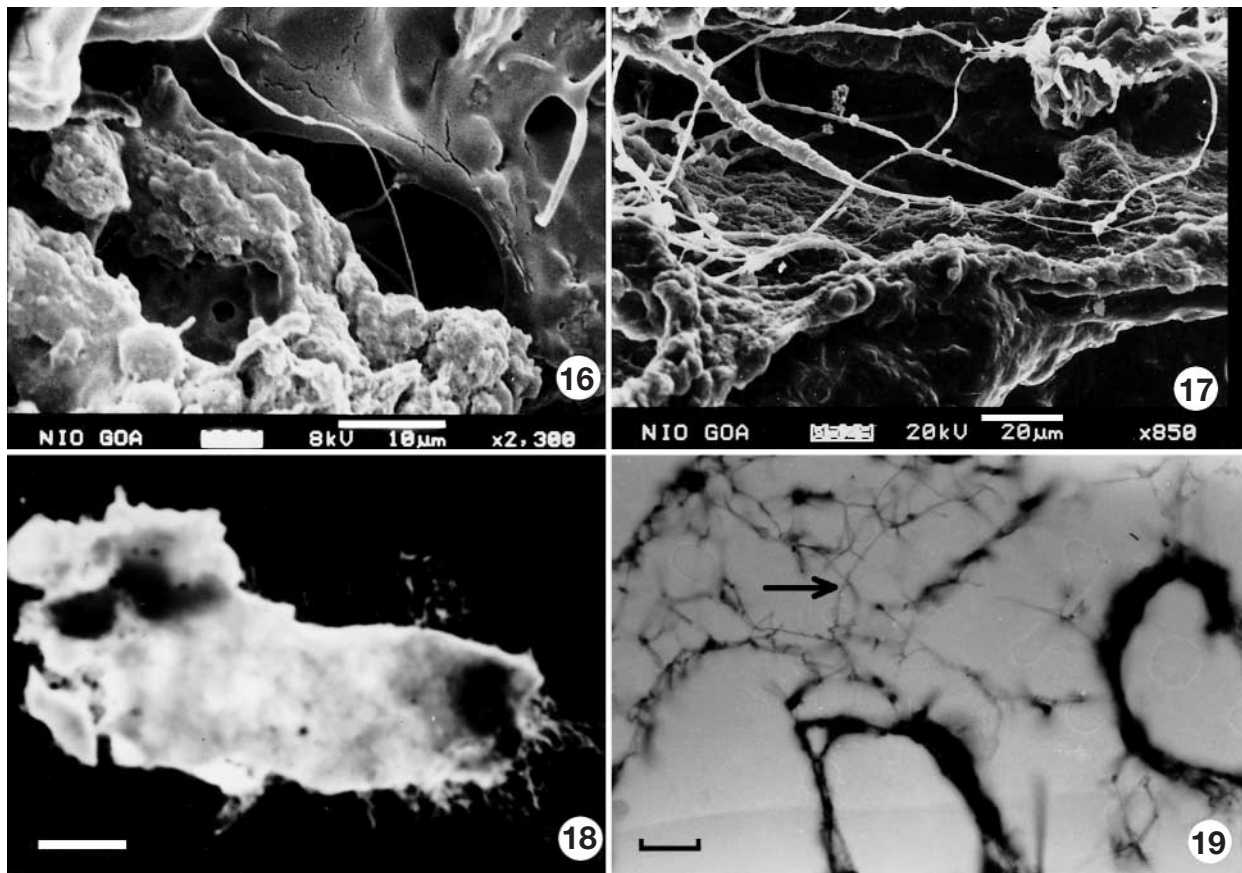
Figs 16 & 17. Fungal hyphae travelling between polyp and carbonate skeleton. Fig. 18. Fungal hyphae around a polyp picked up from the pink line. Scale bar = 370 µm. Fig. 19. Fungal hyphae (arrow) seen around a polyp in a transverse section of pink-line syndrome-affected coral. Scale bar = 20 µm

This study reiterates the hypothesis of Raghukumar & Raghukumar (1998, 1999) that terrestrial fungi are components of certain habitats in the sea. HNS forms were also frequent in our isolations. Immunofluorescence technique revealed the frequent presence of 1 of these isolates (# 98 N-18) within corals. The presence of such non-sporulating fungi has also been reported by Kendrick et al. (1982) in healthy corals. The terrestrial fungus *Aspergillus sydowii* causes rapid erosion and death of sea fan corals in the Caribbean (Smith et al. 1996). More recently, Shinn et al. (2000) reported the presence of terrestrial fungi, especially spores in the dust particles that are blown from Africa to the Caribbean, where they settle on the coral and trigger mass mortality in coral reef.

In view of the above, the behaviour and role of fungi within healthy and diseased corals merit detailed investigations. In this study, fungi were found within the calcium carbonate skeleton and around polyps. SEM studies showed entry of fungi from the coral carbonate skeleton into the polyps, the presence of amorphous 'repair carbonate' around the site of fungal penetration

and precipitation of such 'repair carbonate' around the fungal hyphae. Le Campion-Alsumard et al. (1995a) explained this phenomenon of fungal growth and the polyp's defence mechanisms. During their process of penetration of coral skeleton, their role as bioeroding agents has been confirmed (Perkins & Halsey 1971, Zeff & Perkins 1979, Le Campion-Alsumard et al. 1995a,b). In molluscan shells with fungi, 3 times more calcium was leached than in control shells without fungi (Raghukumar et al. 1988). The high abundance and biomass of fungi that we observed in corals indicate their potential importance as bioeroders of calcium carbonate skeletons in the sea. While bioeroding activities of fungi in dead or diseased portions of corals are understandable, the possibility of such an activity in normal, healthy corals appears paradoxical.

Priess et al. (2000) observed formation and germination of a conidium inside the coral skeletons of *Porites lutea*. However, we rarely noticed this behaviour within corals. This observation, plus the fact that non-sporulating forms were common in corals suggests that these fungi might survive and propagate by means

other than spore formation, such as hyphal fragments (Raghukumar & Raghukumar 1999).

The possible pathogenic role of fungi associated with polyps in the pink-line zone of corals could not be unequivocally shown in the present study although dense fungal hyphae were found around polyps. Fungi as pathogenic organisms are described for *Aspegillus sydowii* in sea fans, causing mass mortalities (Smith et al. 1996). Association of an unidentified lower fungus with black-band disease in star corals (Ramos-Flores 1983) and necrotic patches in massive corals (Raghukumar & Raghukumar 1991) has been reported, without proof of their pathogenicity. Further studies are required to examine the role of fungi in coral pathogenicity. One possibility is that normal residential fungal flora, and not necessarily extraneous invaders, may become opportunistic pathogens under climate variability and anthropogenic stress, as reported for mangrove disease, induced by cyanobacterial symbionts (Rützler 1988).

In conclusion, our study substantiates earlier studies on the presence of fungi in corals and shows that fungi are residential flora within corals. The constant presence and pervasive distribution of fungi, and a high biomass within the polyp zone confirm this. Fungi have been reported to be associated with micrite tubules of the Devonian era (Kobluk & Risk 1974) and modern carbonate sediments (Perkins & Halsey 1971, Perkins & Tsentas 1976). Association of fungi with present-day corals need not be the effect of modern anthropogenic effects, but may be the effect of long-term evolutionary association.

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