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Internal mycobiota of marine macroalgae from the Tamilnadu coast: distribution, diversity and biotechnological potential

Trichur S. Suryanarayanan[1,*], Ambayeram Venkatachalam[1], Nagamani Thirunavukkarasu[2], Jagadesan P. Ravishankar[3], Mukesh Doble[4] and Venkatachalam Geetha[4]

[1] Vivekananda Institute of Tropical Mycology (VINSTROM), Ramakrishna Mission Vidyapith, Chennai 600 004, India, e-mail: t_sury2002@yahoo.com
[2] P.G. and Research Department of Botany, Ramakrishna Mission Vivekananda College, Chennai 600 004, India
[3] Department of Plant Biology and Plant Biotechnology, D.G. Vaishnav College, Chennai 600 106, India
[4] Department of Biotechnology, Indian Institute of Technology of Madras, Chennai 600 025, India

* Corresponding author

Abstract

Eleven brown algae, six green algae and eight red algae occurring along the coast of Tamilnadu state, southern India were screened for their fungal endophyte assemblages. The green algae had a low diversity of endophytes but were more densely colonized. The brown algae supported a higher diversity of endophytes. There were a few dominant endophyte species with a wide host range and several with low colonization frequency that were restricted to a few algal species. The endophytes produced bioactive compounds that inhibited bacteria, an alga and a fungus. They also produced antioxidants and insecticidal metabolites.

Keywords: diversity; endophytes; fungal metabolites; macroalgae; marine algae.

Introduction

There are many reports on the association of parasitic and saprobic fungi with marine algae (Kohlmeyer 1968, Kohlmeyer and Volkmann-Kohlmeyer 2003, Zuccaro and Mitchell 2005), but very few on asymptomatic fungal symbionts of seaweeds (Jones et al. 2008). The few reports on these fungi include those of Zuccaro et al. (2003, 2008), Tsuda et al. (2004), Schulz et al. (2008). Although not all seaweeds are plants (brown algae are not members of the plant kingdom, but green and red algae are), we use the term ''endophytes'' in this study to denote the symptomless fungal endosymbionts present in the marine macroalgae. Marine fungal endophytes of seaweeds (especially the marine-derived forms) have received increasing attention as sources of novel natural products (Bugni and Ireland 2004, Zhang et al. 2006, Jones et al. 2008, Raghukumar 2008, Schulz et al. 2008, Kjer et al. 2010). For example, the marine algal endophyte *Drechslera dematioidea* (Bubák *et* Wróblewski) Subram. *et* Jain produces several bioactive compounds including ten new sesquiterpenes (Bugni and Ireland 2004). Jones et al. (2008) stress the need for studying tropical marine algae for their endophytes as few of these seaweeds have been screened. An understanding of the distribution and diversity of endophytes of marine algae is essential for making prudent bioprospecting decisions.

The aim of the present study was to determine the diversity of fungal endophytes of some of the marine macroalgae off the Tamilnadu coast and to conduct a preliminary evaluation of their potential for production of antialgal, antifungal, antibacterial, antiinsect and antioxidant metabolites.

Materials and methods

Collection sites

The 25 seaweed species (11 brown algae, six green algae and eight red algae) were collected from Mandapam (Palk Bay, 9°16′N, 79°7′E), Keezhakarai (Palk Bay, 9°13′N, 78°46′E), Kodiyakkarai (Palk Strait, 10°16′N, 9°49′E) and Kovalam (Bay of Bengal, 8°22′N, 76°59′E) along the coast of Tamilnadu state (Table 1). Fresh thalli without any disease symptoms were collected, brought to the laboratory in sterile polyethylene bags and processed within 24–36 h.

Isolation and identification of endophytes

The macroalgae were washed thoroughly in running tap water and cut into segments of approximately 0.5 cm^2. For each algal species, 100 segments were screened for the presence of endophytes. Initially, the thallus segments of *Sargassum wightii* were subjected to four different surface sterilization procedures to ascertain the most suitable method for isolating fungi from the algae.

Method A: the segments were dipped in 70% ethanol for 5 s followed by immersion in 4% NaOCl for 60 s and washed with sterile distilled water for 10 s (modified after Suryanarayanan et al. 1998).

Table 1 Algal species studied for their fungal endophyte assemblage.

Host	Host code	Family	Collection site
Green algae			
Caulerpa racemosa (Forsskål) J.Agardh	CR	Caulerpaceae	Mandapam
Caulerpa scalpelliformis (R. Brown *ex* Turner) C. Agardh	CA	Caulerpaceae	Mandapam
Caulerpa sertularioides (S.G. Gmelin) M.A. Howe	CS	Caulerpaceae	Mandapam
Halimeda macroloba Decaisne	HM	Halimedaceae	Mandapam
Ulva fasciata Delile	UF	Ulvaceae	Kovalam
Ulva lactuca Linnaeus	UL	Ulvaceae	Mandapam
Brown algae			
Dictyota dichotoma (Hudson) J.V. Lamouroux	DD	Dictyotaceae	Mandapam
Lobophora variegata var. *indica* Umamaheswara Rao	LV	Dictyotaceae	Keezhakarai
Padina tetrastromatica Hauck	PA	Dictyotaceae	Keezhakarai
Padina gymnospora (Kützing) Sonder	PG	Dictyotaceae	Mandapam
Stoechospermum marginatum (C. Agardh) Kützing	SM	Dictyotaceae	Mandapam
Sargassum ilicifolium (Turner) C. Agardh	SI	Sargassaceae	Mandapam
Sargassum sp.	SA	Sargassaceae	Kodiyakkarai
Sargassum wightii Greville	SW	Sargassaceae	Mandapam
Turbinaria conoides (J. Agardh) Kützing	TC	Sargassaceae	Keezhakarai
Turbinaria decurrens Bory de Saint-Vincent	TD	Sargassaceae	Mandapam
Turbinaria sp.	TU	Sargassaceae	Kodiyakkarai
Red algae			
Portieria hornemanii (Lyngbye) P.C. Silva	CH	Rhizophyllidaceae	Keezhakarai
Gelidiella acerosa (Forsskål) Feldmann *et* G. Hamel	GA	Gelidiellaceae	Mandapam
Gracilaria crassa Harvey *ex* J. Agardh	GC	Gracilariaceae	Mandapam
Gracilaria edulis (S.G. Gmelin) P.C. Silva	GE	Gracilariaceae	Mandapam
Gracilaria sp.	GR	Gracilariaceae	Mandapam
Grateloupia lithophila Børgesen	GL	Halymeniaceae	Mandapam
Halymenia sp. 1	H1	Halymeniaceae	Keezhakarai
Halymenia sp. 2	H2	Halymeniaceae	Mandapam

All collection sites are in Tamilnadu state, India.

Method B: the segments were dipped in 70% ethanol for 5 s followed by immersion in sterile distilled water for 10 s (modified after Zhang et al. 2009).

Method C: the segments were dipped in 70% ethanol for 60 s followed by immersion in sterile distilled water 10 s.

Method D: the segments were dipped in 7.5% H_2O_2 for 60 s and washed three times in sterile distilled water (60 s each) (modified after Girlanda et al. 1997).

One hundred sterilized segments from each macroalga were plated on potato dextrose agar (PDA) medium amended with an antibiotic (chloramphenicol 150 mg l^{-1}). Ten segments of each sample were placed on 20 ml of PDA medium in a Petri dish. The Petri dishes were sealed using Parafilm™ (Pechiney Plastic Packaging Company, Chicago, Il, USA) and incubated in a light chamber for four weeks at 26°C (Suryanarayanan 1992). The light regimen was 12 h of light followed by 12 h of darkness. The Petri dishes were observed periodically, and the fungi that grew out from the tissues were isolated and identified. To prevent the rapidly growing fungi from inhibiting slow growing strains, the former were removed following isolation and identification before they made contact with other isolates (Bills 1996). Sterile isolates and yeasts were given codes using culture characteristics, such as colony surface, texture and pigmentation, and treated as morphospecies (Dobranic et al. 1995, Suryanarayanan et al. 1998).

Statistical analysis

The colonization frequency (CF%) of the endophytes was calculated following Hata and Futai (1995).

$$CF\% = \frac{\text{Total no. of segments colonized by an endophyte}}{\text{Total no. of segments screened}} \times 100 \quad (1)$$

The percentage of tissue segments infected (TI%) by endophyte was calculated as follows:

$$TI\% = \frac{\text{Total no. of segments colonized}}{\text{Total no. of segments screened}} \times 100 \quad (2)$$

A diversity index (Fisher's α) and rarefaction curves were calculated using the software Biodiversity Pro (The National History Museum and The Scottish Association for Marine Science).

Antialgal and antifungal metabolites – bioautogram

The endophytes were grown in potato dextrose broth containing XAD™ amberlite™ (Rohm and Hass, Philadelphia, PA, USA) resin beads. XAD is a polystyrene resin that adsorbs small molecular metabolites produced by fungi and facilitates their easy extraction. The XAD was collected and

kept in 30 ml of methanol for 20 min. The XAD was filtered and the methanol extract was collected. The 30 ml methanol extract was concentrated to 1.5 ml in a Rotavapor (Buchi, Postfach, Switzerland) apparatus. The concentrated extract (50 μl) was spotted on a TLC sheet (pre-coated silica gel, layer thickness 0.1 mm, Merck, Darmstadt, Germany). The chromatogram was developed with a dichloromethane: methanol (96:4) solvent system. After drying, the chromato gram was sprayed with a suspension of *Chlorella fusca* Shih. *et* Krauss cells or spores of *Cladosporium cucumerinum* Ellis *et* Arthur in 2% glucose solution as described by Schulz et al. 1995. The chromatogram was incubated for 3–5 days at 26°C in 10:14 h light:darkness (for *C. fusca*) or total darkness (for *C. cucumerinum*). As a control, methanol (50 μl) was run and tested. The appearance of an inhibition zone on the chromatogram indicated the presence of antialgal or antifungal bioactive secondary metabolite(s). Fifty-four endophyte isolates were screened by this bioassay. The methanol extract obtained as mentioned above from each fungus was used in the following insecticidal, antibacterial and anti-oxidant assays.

Insecticidal metabolites

The methanol extract (50 μl) was spotted on a TLC sheet (pre-coated silica gel, layer thickness: 0.1 mm, Merck). The chromatogram was developed with butanol:acetic acid:distilled water (3:1:1) solvent system up to 10 cm length. After the run, the 10 cm chromatogram was divided into 1 cm sections and each section was eluted with methanol (500 μl), evaporated at room temperature, and the residue was dissolved in 500 μl of deionized water. From this, 200 μl were poured in each well of a microtitre plate, and five maggots (Ceratopogonidae larvae) were placed in each well. As a control, methanol was run on a TLC sheet and 200 μl of this were tested. After 3 or 6 h, the activity of the maggots was observed. Two replicates were set up for each extract. Thirty-two endophyte isolates were tested for insecticidal metabolites.

Antibacterial activity

Strains of *Staphylococcus aureus* Rosenbach (NCIM 5021), *Escherichia coli* (Migula) Castellani *et* Chalmers (NCIM 2931) and *Pseudomonas aeruginosa* (Schröter) Migula (NCIM 5029) obtained from the National Chemical Laboratory (NCL) Pune, India, were cultured in Luria-Bertani (LB) broth (Difco, Detroit, MI, USA, 0446-17-3) at 37°C for 24 h (Perez et al. 1990). Sterile Mueller-Hinton agar plates were used for the bioassay. From a 24 h old culture, 1 ml (with about 100 colony forming units ml^{-1}) was spread on nutrient agar medium. Wells 4 mm in diameter were punched in the agar plate. A methanol extract (50 μl) obtained from the culture filtrate of an endophyte was added to each well. As a control, 50 μl of methanol were added to one of the wells in each plate. Plates were incubated at 37°C for 20 h. Extracts producing a clear zone in the turbid agar were recorded as positive. The methanol (control) produced faint inhibition zones of approximately 1 mm in some, but not all, test plates. Extracts that were positive were tested again to confirm activity. Thirty-eight endophyte isolates were tested for antibacterial metabolites.

Hydroxyl radical scavenging assay

The secondary metabolites of the fungal strains were tested in a hydroxyl radical scavenging assay. Methanol extracts (20 μg of the evaporated extract redissolved in 25 μl of methanol) concentration were used for the assays. The scavenging activity of the hydroxyl radical was determined by estimating competition between deoxyribose and polysaccharide samples for the hydroxyl radicals generated from a Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction). The method of Ohkawa et al. 1979 was used for this. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), K_2HPO_4-KOH buffer (20 mM, pH 7.4), EDTA (0.1 mM), H_2O_2 (1.0 mM), ascorbic acid (0.1 mM) and various concentrations of polysaccharide to arrive at a final volume of 1.0 ml. The reaction mixture was incubated at 30°C for 60 min. The formation of thiobarbituric acid reacting substance (TBARS) was read at 532 nm. The hydroxyl radical scavenging activity was determined by comparing the absorbance of controls (OD_c) with treatments (OD_t). Ascorbic acid was used as standard and the percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{OD_c - OD_t}{OD_c} \times 100 \quad (3)$$

Reducing power

Reducing power was determined by the method of Dorman and Hiltunen (2004) with some modifications. Half a millilitre of the above extract was mixed with 2.5 ml of 0.2 mol l^{-1} phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min; 2.5 ml of 10% trichloroacetic acid were added to the mixture, which was centrifuged at 4500 *g* (Eppendorf Centrifuge 5804 R; Eppendorf, Hamburg, Germany) for 10 min. The upper layer obtained after centrifugation was mixed with 2.5 ml of water and 1 ml of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Ascorbic acid was used as standard. Higher absorbance of the reaction mixture indicated greater reductive potential. Twelve endophyte isolates were screened with the hydroxyl radical scavenging and reducing power assays.

DPPH radical scavenging assay

In this method, the commercially available and stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl), which is soluble in methanol, was used (Aquino et al. 2001). An aliquot (25 μl) of an endophyte culture extract was added to 1 ml of freshly prepared DPPH solution. Absorption was measured at 515 nm after 20 min of incubation. Ascorbic acid used as a standard. All tests were performed in triplicate. Percent inhibition was calculated using Equation 3. Nine endophyte isolates were screened for DPPH radical scavenging assay.

We deposited the following cultures at the Institute of Microbial Technology, Chandigarh, India: MTCC10337 (*Curvularia* sp. isolated from *Sargassum wightii*), MTCC10338 [*Drechslera papendorfii* (Van der Aa) M.B. Ellis comb. nov. isolated from *Turbinaria* sp.], MTCC10339 (*Colletotrichum* sp. isolated from *Sargassum* sp.), MTCC10340 (*Curvularia tuberculata* Jain isolated from *Turbinaria* sp.), MTCC10344 (*Trichoderma* sp. isolated from *Sargassum wightii*), MTCC10345 (*Phomopsis* sp. isolated from *Portieria hornemanii*) and MTCC10346 (*Aspergillus terreus* Thom. isolated from *Ulva lactuca*).

Results

Diversity and distribution

Initially, four different surface sterilization protocols were tested to choose a procedure that would yield the maximum number of endophytes from algal tissues. One hundred tissue segments (0.5 cm^2) of *Sargassum wightii* were surface sterilized by each of the methods detailed above and plated on agar medium to observe the growth of endophytes. Methods A, C and D were less efficient than Method B as they visibly damaged tissues, and the recovery of fungi was low in comparison with Method B. Therefore, this method of sterilization was chosen for further work. The efficacy of B in sterilizing the surface of all the algal species was ascertained by the leaf imprint method (Schulz et al. 2008). Similarly, there was no significant difference between the CF% of endophytes from *S. wightii* on PDA medium and PDA medium made up with 50% seawater. Hence, PDA medium was used.

All the algae screened harboured endophytes; a total of 72 species of fungi was isolated. The density of endophyte colonization varied with the algal host. The TI% colonization varied from 9 in *Gelidiella acerosa* and *Gracilaria* sp. to 100 in *Ulva lactuca* (Table 2). The maximum number of endophyte isolates recovered (from 100 segments) was 155 from *Ulva lactuca*. This was due to multiple infections of this alga by endophytes. The number of endophyte species encountered varied from 2 in *Sargassum ilicifolium* and *Gracilaria crassa* to 25 in *Turbinaria* sp. (Table 2). The species diversity of the endophyte assemblage was lowest for *Sargassum ilicifolium* (0.4) and maximum for *Gelidiella acerosa* (34.6).

The genus *Aspergillus* dominated the endophyte assemblage in 15 of 25 algae studied. Of the three species of *Aspergillus*, *A. terreus* was most frequently isolated from ten algal species and was isolated from all the algae except *Gracilaria crassa* (Table 2). The CF% of yeast sp.1 was high in six algal hosts. Some species of endophytes, such as *Acremoniella* sp., *Aphanocladium* sp., *Ascotricha* sp., *Aspergillus* sp. 10, *Aspergillus* sp. 11, *Aspergillus* sp. 12, *Aspergillus versicolor* (Vuill.) Tiraboschi, *Aureobasidium pullulans* (De Bary) Arnaud, *Chaetomium* sp. 2, *Chaetomium* sp. 3, *Cladosporium* sp. 2, Coelomycete form 1, Coelomycete form 2, *Colletotrichum* sp. 1, *Curvularia* sp. 1, *Drechslera papendorfii* (Van der Aa) M.B. Ellis comb. nov., *Fusarium* sp. 4, *Fusarium* sp. 5, *Memnoniella* sp., *Monilia* sp., *Monodictys* sp., *Mucor* sp., *Myrothecium* sp., *Oidiodendron* sp., *Paecilomyces* sp. 2, *Penicillium* sp. 2, *Phaeotrichoconis* sp., *Phomopsis* sp., *Pseudogymnoascus* like, *Pyrenochaeta* sp., sterile form 12, sterile form 4, *Trichophyton* like, *Trimmatostroma* sp., UNI 2, yeast sp. 3, yeast sp. 4, and yeast sp. 6, were present at low frequency (CF% 2 or less) and were confined to a few algal species (Table 2).

Although the number and size of the tissue segments screened and the surface sterilization procedure and incubation conditions were the same for the different algal species studied, the number of algal species investigated in different seaweed classes screened was different (Table 1). Therefore, to facilitate the comparison of endophyte diversity across algal groups, a rarefaction curve was constructed. This curve is plotted to compare species diversity when sample size differs (Magurran 2004). It showed that the brown algae supported a higher diversity of endophytes, and the green algae harboured the least since the 281 isolates of endophytes recovered from brown, red and green algae had 43.0, 27.7 and 19.4 species, respectively (Figure 1). However, the green algae were more densely colonized by endophytes, as revealed by the TI% index (Table 2).

Bioactive metabolites

The culture extracts of 54 endophyte isolates were tested for their antialgal and antifungal activity. Autobiograms showed that 29 fungi produced metabolites that were either antialgal or antifungal or both; 27 isolates produced antialgal chemicals and eight isolates elaborated antifungal metabolites (Table 3). Of the 19 *Aspergillus* isolates, 12 were positive for antialgal activity; of the 32 isolates tested for antiinsect metabolites, nine produced metabolites that brought about 100% death of the maggots (Table 4). Of the 38 isolates tested for antibacterial activity, 31 inhibited at least one bacterial species (Table 3). Ten isolates produced metabolites that inhibited all three test bacteria (Table 3). *Staphylococcus aureus* was inhibited by metabolites of 26 isolates, *Pseudomonas auregenosa* by 23 isolates and *Escherichia coli* by 18 isolates.

The reducing power (antioxidant activity) of the different fungal species is shown in Table 5. Sterile form 1 and *Aspergillus* sp. 2 had high DPPH radical scavenging activity, while *Curvularia* sp. 1 had good hydroxyl scavenging activity. *A. terreus* from three different algal hosts had good antioxidant activity in all the three assays. *Aspergillus niger* Van Tieghem, *A. terreus*, *Curvularia* sp. 1, *Paecilomyces* sp. 1 and *Penicillium* sp. 1 had good total reducing power. Thus, secondary metabolites isolated from marine endophytic fungi have reducing power activity.

Discussion

The genus *Aspergillus* (especially *A. terreus*) dominated the endophyte assemblages of several algae screened (Table 2). Many species of the genus *Aspergillus* are halotolerant (Gunde-Cimerman et al. 2009). *A. terreus* is known to occur

Table 2 CF% {[(Total no. of segments colonized by an endophyte)/(total number of segments screened)]×100} of endophytic fungi isolated from different marine macroalgae (for host code, refer to Table 1).

Fungus	Green algae						Brown algae											Red algae							
	CR	CA	CS	HM	UF	UL	DD	LV	PA	PG	SM	SI	SA	SW	TC	TD	TU	CH	GA	GC	GE	GR	GL	H1	H2
Acremoniella sp.									2																
Alternaria sp. 1											1					1			1						
Alternaria sp. 3																	29								
Alternaria sp. 4																	3								
Alternaria sp. 5													1				4								
Aphanocladium sp.																						1			
Ascotricha sp.									1																
Aspergillus niger	2	1	1	12	2	9	3	12	16*				7	3					2*			3*	4	6*	12
Aspergillus sp. 2	4	1	1	4	1	46			1					1			2	1					1	3	26*
Aspergillus sp. 4	2								1								1								
Aspergillus sp. 5				1					1						1										
Aspergillus sp. 10														1											
Aspergillus sp. 11														1											
Aspergillus sp. 12																	1								
Aspergillus terreus	1	43*	2	66*	7*	86*	1	27*	9	8*	4*	73*	3	3	2	2	4	5*	1		75*	2	6	2	19
Aspergillus versicolor																			1						
Aureobasidium pullulans	1																								
Chaetomium sp. 1	1		1		1	1		2	1	1	2				1	2							1	1	
Chaetomium sp. 2															1	1									
Chaetomium sp. 3																1	1								
Cladosporium sp. 1	7		1			3				1				1	1		3	4					1	1	1
Cladosporium sp. 2										1															
Cladosporium sp. 3																	4								
Coelomycete form 1																								1	
Coelomycete form 2														1											
Colletotrichum sp. 1													1				1								
Curvularia lunata (Wakkar) Boedijin					1								1				7								
Curvularia sp. 1		1														1									
Curvularia sp. 3					1												5								
Curvularia tuberculata																	5								
Drechslera papendorfii																	1								
Drechslera sp. 1													2			1	13								2
Emericella nidulans (Eidam) Vuill.								2			1				1	2	1	1					1	1	1
Fusarium sp. 2	5		1																						
Fusarium sp. 4	1																								
Fusarium sp. 5	1																1								
Helicosporium sp.													9												
Memnoniella sp.																		2							
Monilia sp.																						1			
Monodictys sp.									1								1								
Mucor sp.	1																								
Myrothecium sp.			1																						

(Table 2 continued)

Fungus	Green algae						Brown algae											Red algae							
	CR	CA	CS	HM	UF	UL	DD	LV	PA	PG	SM	SI	SA	SW	TC	TD	TU	CH	GA	GC	GE	GR	GL	H1	H2
Nigrospora sp. 1						2		2			2		1	1		4			1		1		1		
Oidiodendron sp.													1												
Paecilomyces sp. 1		10		6	2				1					1			1				1			2	
Paecilomyces sp. 2																						1			
Penicillium sp. 1	22	8	1	3		8			5	2			2	1				1	1						5
Penicillium sp. 2																		1							
Phaeotrichoconis sp.																	2								
Phialophora sp.			1						2							1									
Phoma sp.	1									1					2				1					1	
Phomopsis sp.																		2							
Pseudogymnoascus-like																1									
Pyrenocheta sp.	1																								
Sterile form 1	1		2				1			2		4	2	1		12*	2	1	1	2					
Sterile form 2									1	1			1	2	1										
Sterile form 3					2			2			1				6*								1		
Sterile form 4													2												
Sterile form 12													1												
Taeniolella sp.													7												
Trichoderma sp.				1			22*			1										32*					
Trichophyton like																		1							
Trimmatostroma sp.																		1							
UNI 1								3																	
UNI 2																2									
UNI 3																	3								
Varicosporium sp.													44*				3								
Yeast sp. 1	65*		69*	6	7*								5	32*			36*						11*		27
Yeast sp. 2																		2				1			
Yeast sp. 3														1											
Yeast sp. 4																		1							
Yeast sp. 6					1																				
Total no. of species	16	6	11	8	10	7	4	7	13	9	6	2	17	14	9	13	25	13	8	2	3	6	9	9	8
Total no. of isolates	116	64	81	99	25	155	27	50	42	18	11	77	90	50	16	31	134	23	9	34	77	9	27	18	93
Fisher's α	5	1.6	3.4	2.1	6.2	1.5	1.3	2.2	6.4	7.2	5.4	0.4	6.2	6.5	8.5	8.4	9.1	12	35	0.5	0.6	7.9	4.7	7.2	2.1
TI%	91	64	81	88	23	100	27	46	42	18	11	77	71	50	16	31	99	23	9	34	77	9	27	18	92

*Fungus with highest CF%.

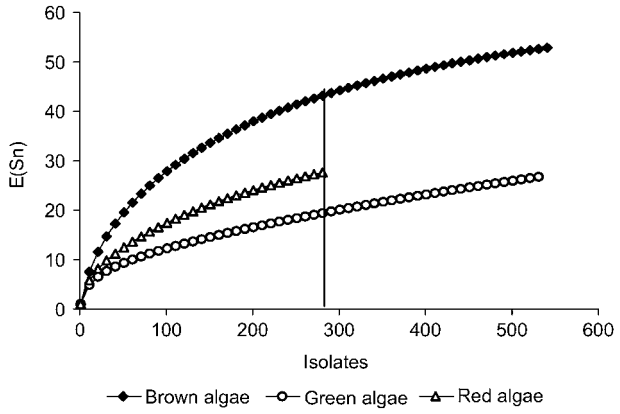

Figure 1 Rarefaction curve showing expected number E(Sn) of endophyte species.
Rarefaction curves were drawn by repeatedly sampling all of the recorded species at random (Gotelli and Colwell 2001). The curves predict the expected number of species from a collection of random samples.

in various marine habitats including marine algae (Lee et al. 2003), hypersaline waters of the Dead Sea (Kis-Papo et al. 2003), and deep seas (Damare et al. 2006). It is the most frequent fungal associate of soft corals and sponges (Kamat et al. 2008). According to Höller et al. (2000), the genus *Aspergillus* predominates in the sponge genus *Ircinia* off Malta. The dominance of *Aspergillus* spp. as endophytes (which are only sporadically isolated as endophytes from tissues of terrestrial plants) and the absence of dominant, generalist endophytes of terrestrial plants, such as *Phyllosticta* spp. (Pandey et al. 2003, Okane et al. 2003, Motohashi et al. 2009), *Pestalotiopsis* spp. (Jeewon et al. 2004, Tejesvi et al. 2009, Romina and Priscila 2010), *Phomopsis* spp. (Murali et al. 2006) and *Colletotrichum* spp. (Lu et al. 2004) in algae suggest that *Aspergillus* species are adapted to survive as endophytes in marine algae and have possibly coevolved with the algae. It is pertinent to note here that Schulz et al. (2008) also noticed that the dominant endophyte species isolated from terrestrial plants and fungi (not necessarily endophytes) associated with marine algae differed. The observation that yeast sp. 1 was dominant in a few macroalgae is interesting as a similar result was obtained for marine macroalgae from Antarctica by Loque et al. (2010).

The endophyte assemblage of the algae comprised a few dominant species and many less frequently (although not unique) encountered ones. This is similar to the pattern of endophyte species composition of terrestrial plant communities (Suryanarayanan et al. 2003, Saikkonen 2007). Interestingly, *Aspergillus terreus* had a wide host range and was not restricted to any group of algae; it was isolated as an endophyte from brown, green and red algae. Such a loose host affiliation of the dominant endophyte species is similar to that among endophytes of terrestrial plants (Cannon and Simmons 2002, Suryanarayanan et al. 2003). Molecular evidence obtained recently by Harvey and Goff (2010) also suggests that certain endophytes of marine algae may have loose host affiliation. Marine macroalgae secrete metabolites that inhibit colonization of the thalli by fungi (Fletcher 1975, Zuccaro and Mitchell 2005). Studies are needed to correlate the sensitivity of different fungal species to such metabolites as differential sensitivity among fungi may possibly explain the existence of a core group of fungal species as endophytes in marine algae.

Among the algal groups screened, the green algae harboured a low diversity of endophytes. One of the reasons for this could be the short life cycles of some species in this group coupled with the slow growth that is characteristic of the fungal endophytes (Zuccaro and Mitchell 2005). It is well known that marine substrates of the tropics support ubiquitous fungal genera of terrestrial origin, such as *Aspergillus* (Höller et al. 2000, Bugni and Ireland 2004, Proksch et al. 2008). These so called marine-derived fungi possibly moved from terrestrial habitats to marine habitats (Proksch et al. 2008). These fungi are prolific producers of novel bioactive compounds perhaps as a result of their adaptation to the marine environment (Proksch et al. 2008). The diversity of foliar endophytes of terrestrial plants is known to change with tissue age and season (Photita et al. 2004, Suryanarayanan and Thennarasan 2004). Hence, more detailed studies involving different parts and age levels of thalli as well as sampling during different seasons are needed to determine whether the species composition of endophytes is dynamic and whether true marine fungi survive as endophytes in these algae. It would also be worthwhile to screen the algae using molecular probes specific to fungi (Zuccaro et al. 2008), though differentiating surface colonizers from endophytes will be a daunting task.

The role of endophytes in ecosystems is not clearly known. Endophytes of terrestrial plant communities possibly initiate litter degradation (Kumaresan and Suryanarayanan 2002, Osono 2002, Yanna et al. 2002, Hyde et al. 2007), and probably increase host fitness by deterring insect pests (Akello et al. 2007) and pathogens (Arnold et al. 2003). It will be worthwhile addressing topics, such as the role of algal endophytes in detritus decomposition, and defence against pathogens.

Terrestrial endophytes are a rich source of bioactive compounds, including novel antibiotics, anti cancer metabolites and anti oxidants (Strobel and Daisy 2003, Gunatilaka 2006, Huang et al. 2007, Suryanarayanan et al. 2009, Weber 2009). Marine-derived fungi are currently being vigorously screened for bioactive compounds as they produce several novel chemicals (Liu et al. 2006). For example, 27% of the new compounds obtained from the marine derived fungi come from those associated with algae (Bugni and Ireland 2004). Some of these fungi produce compounds possessing totally novel carbon frameworks (Kjer et al. 2010). In the present study, *Aspergillus terreus* isolated as endophyte from *Halimeda macroloba*, *Gracilaria edulis* and *Ulva lactuca* produced metabolites that were active against bacteria, algae and fungi (Table 3). This fungus isolated from the tissues of *Caulerpa scalpelliformis*, *Caulerpa sertularioides*, *Gracilaria edulis*, *Sargassum ilicifolium* and *Ulva lactuca* also produced insecticidal compounds (Table 4). The same species of endophytes isolated from different algal host showed variation in

Table 3 Antibiotic, antialgal and antifungal activity of culture extracts of fungi (endophytes) from marine macroalgae (for host code, refer to Table 1).

Fungus	Host code	Antibiotic activity			Antialgal activity	Antifungal activity
		SA[1]	PA[2]	EC[3]	CF[4]	CC[5]
Alternaria sp.	SW	a	a	–	–	–
Aspergillus niger	CA	–	–	–	–	–
Aspergillus niger	CR	*	*	*	–	–
Aspergillus niger	CS	*	*	*	–	–
Aspergillus niger	HM	a	a	a	+	–
Aspergillus niger	UL	*	*	*	+	–
Aspergillus sp. 2	CA	a	b	–	–	–
Aspergillus sp. 2	HM	a	a	–	+	–
Aspergillus sp. 2	SW	–	–	–	–	–
Aspergillus sp. 2	UL	*	*	*	–	–
Aspergillus sp. 4	CR	*	*	*	+	–
Aspergillus sp. 4	SW	b	–	–	+	–
Aspergillus sp. 5	HM	*	*	*	+	–
Aspergillus sp. 10	SW	a	–	–	–	–
Aspergillus terreus	CS	*	*	*	+	+
Aspergillus terreus	CA	a	a	a	+	–
Aspergillus terreus	GE	a	a	b	+	+
Aspergillus terreus	HM	*	*	*	+	+
Aspergillus terreus	SI	*	*	*	+	–
Aspergillus terreus	UL	a	a	a	+	+
Chaetomium sp.	UL	*	*	*	+	–
Chaetomium sp. 1	H1	a	a	a	+	+
Chaetomium sp. 1	PT	*	*	*	+	+
Cladosporium sp. 1	CH	a	b	–	–	–
Cladosporium sp. 1	SW	–	c	c	–	–
Cladosporium sp. 1	UL	*	*	*	–	–
Cladosporium sp. 1	CR	–	a	a	–	–
Curvularia sp. 1	SW	–	–	–	–	+
Curvularia sp. 1	CA	–	–	–	–	–
Curvularia sp. 3	SW	a	b	b	–	–
Emericella nidulans	CH	–	–	–	–	–
Emericella nidulans	H1	b	–	–	–	–
Emericella nidulans	SW	a	–	–	–	–
Fusarium sp.	SW	a	a	a	+	–
Fusarium sp. 2	CS	a	a	a	+	–
Fusarium sp. 4	CR	*	*	*	–	–
Nigrospora sp.	GE	–	–	c	+	–
Nigrospora sp.	UL	*	*	*	+	–
Paecilomyces sp. 1	CA	a	a	–	+	–
Penicillium sp.	CH	a	b	–	–	–
Penicillium sp. 1	CA	a	a	a	+	–
Penicillium sp. 1	CR	a	a	a	–	–
Penicillium sp. 1	CS	a	a	a	–	–
Penicillium sp. 1	HM	a	a	a	+	–
Pestalotiopsis sp. 3	SW	–	–	–	–	–
Phomopsis sp.	CH	–	–	c	–	–
Sterile form 1	CH	a	–	b	+	–
Sterile form 1	CR	–	c	c	–	–
Sterile form 1	SW	–	–	–	–	–
Sterile form 2	SW	a	a	–	+	–
Sterile form 3	LV	a	–	–	+	–
Sterile form 3	TC	*	*	*	–	–
Trichoderma sp.	HM	*	*	*	+	–
Trichoderma sp.	SW	b	a	–	+	+

[1] *Staphylococcus aureus*; [2] *Pseudomonas aeruginosa*; [3] *Escherichia coli*; [4] *Cholorella fusca*; [5] *Cladosporium cladosporioides*.
a, Obvious inhibition zone (>10 mm in diameter); b, slight inhibition zone (3–10 mm in diameter); c, faint inhibition zone (1–3 mm in diameter); –, no inhibition; +, inhibition; *, not tested.

Table 4 Insecticidal activity of fractions of methanol culture extracts of (endophytes) fungi isolated from marine macroalgae (for host code, refer to Table 1).

Fungus	Host code	% Death of maggots with different fractions (200 μl) of TLC of methanol culture extract									
		1	2	3	4	5	6	7	8	9	10
Control		*	*	*	*	20	*	*	*	*	*
Aspergillus niger	CA	*	*	*	*	*	*	*	*	*	*
Aspergillus niger	CR	*	*	*	*	*	20	*	*	*	*
Aspergillus niger	CS	*	*	*	*	*	*	*	*	*	*
Aspergillus niger	HM	*	*	*	*	*	*	*	*	*	*
Aspergillus niger	UL	*	*	*	*	60	*	*	*	*	*
Aspergillus sp. 2	CA	*	*	*	*	*	*	*	*	*	*
Aspergillus sp. 2	HM	60	*	*	*	*	*	*	*	*	*
Aspergillus sp. 2	UL	*	*	*	*	*	*	*	*	*	*
Aspergillus sp. 4	CR	*	*	*	*	*	*	*	*	*	*
Aspergillus terreus	CA	*	*	*	*	*	*	*	*	100	*
Aspergillus terreus	CS	20	*	*	*	*	*	*	*	100	*
Aspergillus terreus	GE	*	*	*	*	*	*	*	100	100	*
Aspergillus terreus	HM	20	*	*	*	*	*	*	*	20	*
Aspergillus terreus	SI	*	*	*	*	*	*	*	40	100	*
Aspergillus terreus	UL	*	*	*	*	*	*	*	*	*	100
Chaetomium sp. 1	UL	*	*	*	*	20	*	*	*	*	*
Cladosporium sp. 1	CR	*	*	20	*	*	*	*	*	*	*
Cladosporium sp. 1	UL	*	*	*	*	*	*	*	*	*	*
Curvularia sp.	CA	*	*	*	*	*	*	*	*	*	*
Emericella nidulans	CH	*	*	*	*	*	*	*	*	*	*
Fusarium sp. 2	CS	*	*	*	*	*	*	*	*	100	*
Fusarium sp. 4	CR	*	*	*	*	*	*	*	20	*	*
Myrothecium sp.	CR	*	*	*	*	*	*	*	*	*	*
Nigrospora sp.	GE	*	*	*	*	*	*	*	*	*	*
Nigrospora sp.	UL	*	*	*	*	*	*	*	*	*	*
Paecilomyces sp. 1	CA	*	*	*	*	*	*	100	100	*	*
Paecilomyces sp. 1	HM	*	*	*	*	*	*	*	*	*	*
Penicillium sp. 1	CA	*	*	*	*	*	*	*	*	100	*
Penicillium sp. 1	CR	*	*	*	*	*	*	*	*	*	*
Penicillium sp. 1	CS	*	*	*	*	*	*	*	*	*	*
Penicillium sp. 1	HM	*	*	*	*	*	*	100	*	*	*
Sterile form 1	CR	20	*	*	*	*	*	*	*	*	*

*, No activity.

Table 5 Antioxidant activity of the methanol culture extracts of fungi isolated from marine macroalgae (mean of 3 readings±SE; for host code, refer to Table 1).

Fungus	Host code	DPPH radical scavenging assay (%)	Hydroxyl radical scavenging assay (%)	Total reducing power assay (%)
Ascorbic acid control		38.87±3.11	71.49±7.11	16.32±1.30
Aspergillus niger	CA	1.62±0.14	27.99±2.52	34.16±4.10
Aspergillus sp. 2	CA	60.77±6.68	9.81±0.78	21.67±2.37
Aspergillus terreus	CA	21.67±2.16	11.63±1.16	27.96±3.07
Aspergillus terreus	GE	45.84±3.66	18.44±1.84	27.14±2.17
Aspergillus terreus	SI	0.76±0.06	1.17±0.14	46.34±4.61
Aspergillus terreus	UL	*	13.72±1.09	18.71±1.80
Curvularia sp. 1	CA	43.30±3.89	42.54±3.40	48.55±4.33
Fusarium sp. 4	CR	*	1.07±0.04	18.02±1.10
Paecilomyces sp. 1	CA	45.22±3.60	0.99±0.11	43.53±4.33
Penicillium sp. 1	CA	3.17±0.22	0.72±0.58	49.00±3.92
Penicillium sp. 1	HM	*	11.72±0.86	9.56±0.76
Sterile form 1	CR	79.86±6.39	9.06±0.72	19.77±1.58

*, Not tested.

their active metabolites profile thus indicating genetic variation among the endophytes. Seymour et al. (2004) believe that genetically similar endophytes may have significant differences in their metabolic profiles. Thus, although species diversity of macroalgal endophytes may be low due to the occurrence of fungi with wide host ranges, the secondary metabolite diversity may be high. For example, marine derived *A. terreus* strains produce novel butenolides (Parvatkar et al. 2009) and a new UV-A protecting dipyrroloquinone (Lee et al. 2003). Other species of marine-derived *Aspergillus* are prolific producers of novel metabolites (Debbab et al. 2010).

Endophytes of terrestrial plants are a good source of antioxidants (Huang et al. 2007, Yuan et al. 2009) including phenolic acids, flavonoids, tannins, hydroxyanthraquinones and terpenoids, which scavenge superoxide and hydroxyl free radicals (Strobel and Daisy 2003). Our results showed that endophytes of marine algae are also a potential source of antioxidants. White and Torres (2010) suggest that endophytes protect their host plants from diseases, drought and heavy metal toxicity by producing antioxidant chemicals, thereby increasing tolerance of the host to oxidative stress. Further studies are necessary to determine whether endophytes of marine macroalgae also confer such benefits to their hosts by enhancing their ability to tolerate oxidative stress.

Conclusion

- Fungal endophyte assemblages of brown algae are more diverse than those of the green algae.
- Some fungal endophyte species have a wide host range.
- The ubiquitous endophytes of terrestrial plants found in this climate zone are not present in the algae tested.
- Algal endophytes are a good source of bioactive metabolites, such as antioxidants, antibiotics, antialgal, antifungal and antiinsect compounds.

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