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Diversity and potential antifungal properties of fungi associated with a Mediterranean sponge

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Abstract Fungi that inhabit marine sponges occupy an ecological niche that has recently attracted great attention due to the potential in either ecological or pharmaceutical advances. The ecological interaction between marine sponges and fungi is, however, only poorly understood. Eighty five fungal taxa were isolated from the marine sponge *Psammocinia* sp. from the Mediterranean Sea. The majority (89%) of these taxa were isolated using a `sample compressing` method, in combination with the use of fungicides-amended medium. Abundant `terrestrial` taxa such as *Acremonium*, *Penicillium* and *Trichoderma* were found along with potentially undescribed *Phoma* and *Trichoderma* species. Several of these taxa exhibited in vitro anti-fungal properties as determined against four test fungi. Even though a significant number of fungal taxa were isolated during this study, we estimate that the diversity of fungi that are associated with *Psammocinia* sp. is higher than reported here. It is advocated that *Psammocinia*, and other sponge genera, may be a prime niche for discovering new fungal species as well as novel anti-fungal compounds from fungal sources.

Keywords *Acremonium* · *Aspergillus* · Fungicides · Marine fungi · *Penicillium* · *Phoma* · *Psammocinia* · *Trichoderma*

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Introduction

Since the pioneering work of Barghoorn and Linder (1944) significant progress has been made on the observation and identification of marine-derived fungi (Hyde et al. 2000, Kohlmeyer and Kohlmeyer 1979; Jones et al. 2009). Many marine fungi collected directly on substrates from the marine milieu are unique and have been shown to be phylogenetically distant from most terrestrial species (Vijaykrishna et al. 2006; Jones et al. 2009). Many fungi isolated from various marine habitats using culture based technology, however, have also been found to be taxonomically closely related to species from genera that are well known from terrestrial environments, such as *Acremonium, Aspergillus, Penicillium* and *Trichoderma* (Höller et al. 2000, Wang et al. 2008; Raghukumar 2008; Jones et al. 2009).

Marine sponges (Porifera) are significant constituents of both coastal and deep sea environments (Bergquist 1978; Hentschel et al. 2003) and are known to harbor a wide

diversity of microorganisms (Tsoukatou et al. 2002; Taylor et al. 2007a, b). These associated microorganisms are thought to be involved in a variety of ecological functions including production of secondary metabolites that can contribute to their own ecological success, and to that of their host (Höller et al. 2000). A similar situation is thought to occur when plants are colonized by internal fungi (Kumar and Hyde 2004). Some of the chemicals produced by these microorganisms have been shown to exhibit anti-predation, anti-competition and anti-fouling capabilities (Hay 1996, Lopanik et al. 2004).

The study of sponge-associated fungi has gained momentum in recent years, partly due to an increased drive to find new natural products from marine microbial sources (Morrison-Gardiner 2002; Bugni and Ireland 2004), in a manner that is also being undertaken in conjunction with the study of plant associated fungi (Kumar and Hyde 2004; Huang et al. 2008). Numerous new compounds and antibiotics have already been discovered from internal fungi isolated from marine organisms (Raghukumar 2008), as is also the case for plant-associated fungi (e.g. esters, alcohols, and small molecular weight acids: Mitchell et al. 2008).

Information concerning the diversity of sponge-associated fungi has focused on wide scale isolation of fungal strains from various sponge species, and has contributed to the assessment of fungal diversity between sponge species. However, studies concerning the diversity of sponge-associated fungi within a single marine sponge species or genus are more limited (Gao et al. 2008; Proksch et al., 2008; Wang et al. 2008).

Most studies to date have utilized plating-based techniques for fungal isolation or have based much of their analysis on DNA samples extracted from the sponge. Though plating can be effective, this method may not necessarily yield the highest number and/or broadest diversity of microorganism propagules per sample volume (Höller et al. 2000).

The present study focused on isolation of fungi from specimens of the Mediterranean sponge genus *Psammocinia* (Irciniidae) by employing different methodologies than are usually used. This genus was studied because of the known high diversity of its associated microorganisms (Weisz et al. 2007, 2008). An initial survey indicated that this is a common sponge species along the Eastern Mediterranean coast and is found in relatively shallow water. The diversity of fungi associated with this sponge species and the prevalence of their diffusible anti-fungal characteristics were also assessed. The effects of sponge sample compression and antibiotic amendments to the isolation medium on the isolation of *Psammocinia*-associated fungi were assessed. Lastly the potential of some marine isolates to mycoparasitise other fungi isolated from the same marine environment was evaluated.

Materials and methods

Study area and sampling method

Samples of the sponge *Psammocinia* sp. were collected using SCUBA diving at a depth of 2–6 m, approximately 200 m off-shore at Sedot-Yam, Israel (North 32° 29′, East 34°53′). Sponge samples were collected in the winter (January 2007) and summer (June 2007), at the same location (N=5 at each sampling date). Sediment and sea-water were sampled from the same site as controls. Samples were kept in sealed plastic bags containing sea water and processed within 2–3 hours after sampling. The sponge samples (ca. 5×5 cm) were rinsed twice in sterile double distilled water prior to further analyses.

Isolation techniques and culture media

To determine whether the variety of isolated fungi can be increased, the standard isolation medium, potato dextrose agar (Difco) with 250 mg/l chloramphenicol (PDACl) was amended with different fungicides, listed in Table 1. Two methods were used to isolate fungi: (1) direct plating of inner sponge tissue fragments (ca. 1 cm^3) on Petri dishes (Ø=90 mm) containing the media amended with the difference fungicides (Table 1) and (2) plating of 200 microliters/dish of sponge extract obtained by compressing the sponge samples (ca. 1 cm^3) using a sterile mortar and pestle. The Petri dishes were incubated at 25°C in the dark for 3–30 days. Edges from emerging fungal colonies were transferred, repeatedly, to fresh PDACl plates to obtain pure cultures.

Molecular identification of the fungal strains

Initial molecular identification was based on ITS sequences. Fungal genomic DNA was extracted from 15–20 mg of ground lyophilized mycelia as described by Paz et al. 2007. The DNA (50–300 ng/µl) of each strain was used as a template for PCR, using ITS1 and ITS4 primers (White et al. 1990). Cycling parameters were: initial denaturation at 94°C for 6 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 seconds, and 70°C for 40 s. The reaction was terminated following a final elongation step of 7 min at 70°C. For additional sequence data required for identification within some specific genera, segments of the following genes were amplified and sequenced: β-tubulin (for *Penicillium* spp. according to the methodology described in Samson et al. (2004)); partial translation elongation factor 1-α (*tef1*) (for *Fusarium* spp., Geiser et al. 2004; or for *Trichoderma* spp. Druzhinina et al. 2008); and actin (for *Cladosporium* spp., Wirsel et al. 2002). Alignments and phylogenetic analyses of sequence data were performed using Molecular Evolutionary

Table 1 Antifungal compounds used in this study

Generic name	Chemical name	Source and concentrations used
Folpet	N-[(Trichloromethyl)thio]phthalimide	Folpan, Makhteshim-Agan Group, Israel, 1–10 μg/ml
Prochloraz	N-propyl-N-[2-(2,4,6-trichlorophenoxy) ethyl]imidazole-1-carboxamide	Mirage, Makhteshim-Agan Group, 1–10 μg/ml
Guanidine	N,N‴-(iminodi-8,1-octanediyl)bis[guanidine] and ddditional polyamines	Panoctine, Makhteshim-Agan Group, 1–10 μg/ml
Tebuconazole	(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol	Orius, Makhteshim-Agan Group, 1–10 μg/ml
Imazalil	1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl}-1H-imidazole	Magnate, Makhteshim-Agan Group, 1–10 μg/ml
Captan	3a,4,7,7a-tetrahydro-2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione	Merpan, Makhteshim-Agan Group, 1–10 μg/ml
Bupirimate	butyl-2-ethylamino-6-methylpyrimidin-4-yl dimethylsulfamate	Nimrod, Makhteshim-Agan Group, 1–10 μg/ml
Benomyl	Methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate	Benlate, DuPont, USA, 1–10 μg/ml
Kresoxim-methyl	methyl (*E*)-methoxyimino[α-(*o*-tolyloxy)-*o*-tolyl]acetate	Ardent, Makhteshim-Agan Group, 1–10 μg/ml
PCNB	Pentachloronitrobenze	PCNB, Luxemburg chemicals, Israel, 1–10 μg/ml
Carboxine	5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide	Carboxine, Dushefa Biochemie B.V., The Netherlands, 200 μg/ml
Hygromycin B	*O*-6-Amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1-2-3)-*O*-β-D-talopyranosyl(1-5)-2-deoxy-N^3-methyl-D-streptamine	Hygromycin B, Sigma Aldrich, USA, 100 μg/ml
Cycloheximide	4-{(2R)-2-[(1S,3S,5S)-3,5-dimethyl-2-oxocyclohexyl]-2-hydroxyethyl}piperidine-2,6-dione	Cycloheximide, Sigma Aldrich, 500 μg/ml

Genetics Analysis (MEGA) software version 4.0 (Tamura et al. 2007). Neighbor-Joining phylogenetic analysis was evaluated by 1,000 bootstrap replicates (Hillis and Bull 1993).

Antifungal and mycoparasitic assays

For detecting diffusible anti-fungal properties, a PDACl plug (Ø=10 mm) of each collected strain was placed on a broad disk of pre-sterilized dialysis tubing placed (MWCO-12-14000 Daltons; Medicell, UK), atop fresh PDACl in a standard Petri dish. The fungi were cultured at 25°C for 5–10 days (depending on growth rates of the different isolates), after which the dialysis tubing (along with the culture) was removed. To determine the presence of secreted anti-fungal compounds, three test fungi, viz. *Alternaria alternata* (Aa01), *Pythium aphanidermatum* (BK11) and *Rhizoctonia solani* (TP6) were obtained from the Dept. of Plant Pathology and Microbiology and *Neurospora crassa* (74-OR23-1A) from The Fungal Genetics Stock Center. These test fungi were placed on the Petri dish and the effect of diffused compounds from the marine isolate was assessed (Morton and Stroube 1955). The formation of inhibition zones wider than 15 mm between the source, which was placed in the center of the dish and the test organism -placed nearer to the dish edge- was considered as an indicator for antagonism. Mycoparasitic properties of all *Trichoderma* spp. isolated from *Psammocinia* specimens were studied by microscopic observations (following staining with cotton blue in lactophenol) using *Aspergillus* spp. (strains OY207, OY907, OY31107 and OY34807) and *Fusarium* spp. (strains OY2107, OY11007 and OY32307) as hosts cultured on either PDACl or sea water agar.

Results

Throughout this study, ten sponge samples were analyzed, yielding over 400 individual fungal colonies. Two hundred and twenty isolates were subjected to molecular identification based on amplification of the ITS (internal transcribed spacers 1 and 2) of the rRNA gene cluster 85 taxa were identified on the basis of comparison to GenBank data (Table 2). The majority of these were isolated by using the "sponge compressing" method (n_{sc}=76), while the traditional 'plating method' yielded only about 10% (n_{pm}=9) of the fungal isolates obtained (Fig. 1). Among the isolated

Table 2 Fungal taxa found in association with *Psammocinia* sp.

Phylum	Taxon	Strain no.	[1]GenBank Accession No.	Inhibitory activity
Ascomycota	*Acremonium implicatum*	OY36707	FJ571419	–
	Acremonium sp.	OY12307	FJ571422	–
	Acremonium sp.	OY1707	FJ571421	–
	Acremonium sp.	OY2507	FJ571420	–
	Acremonium sp.	OY34007	FJ571423	–
	Alternaria sp.	OY14007	FJ571424	–
	Ascomycete	OY16007	FJ571425	–
	Ascomycete	OY17007	FJ571426	+
	Ascomycete	OY18707	FJ571427	+
	Ascomycete	OY3007	FJ571431	–
	Ascomycete	OY19307	FJ571428	+
	Ascomycete	OY19907	FJ571429	+
	Ascomycete	OY20707	FJ571430	+
	Ascomycete	OY40007	FJ571432	+
	Aspergillus sp.	OY10907	FJ571435	+
	Aspergillus sp.	OY35807	FJ571436	+
	Aspergillus sp.	OY907	FJ571433	+
	Aspergillus sydowii	OY34807	FJ571437	+
	Aspergillus terreus	OY9107	FJ571438	+
	Aspergillus ustus	OY207	FJ571439	+
	Aspergillus versicolor	OY31107	FJ571440	–
	Bionectria pseudochroleua	OY10207	FJ571441	+
	Chaetomium sp.	OY33207	FJ571443	+ (–P)
	Cephalosporium sp.	OY16607	FJ571442	–
	Cladospotium oxysporum	OY107	FJ571444, [a]FJ619245	–
	Cladosporium sp.	OY1807	FJ571445, [a]FJ619246	–
	Cladosporium tenuissimum	OY607	FJ571446, [a]FJ619247	–
	Clavicipitace	OY39207	FJ571447	–
	Cochliobolus sp.	OY32107	FJ571448	–
	Didymella sp.	OY6207	FJ571449	+
	Dothideomycetes sp.	OY307	FJ571450	+
	Emericellopsis sp.	OY1407	FJ571451	–
	Eupenicillium sp.	OY30107	FJ571452	–
	Fusarium equiseti	OY15407	FJ571454, [c]FJ619270	–
	Fusarium proliferatum	OY2107	FJ571455, [c]FJ619268	–
	Fusarium solani	OY32307	FJ571456, [c]FJ619269	–
	Gliomastix sp.	OY17407	FJ571457	–
	Gymnoascus sp.	OY18207	FJ571458	–
	Hypocrea orientalis	OY2607	FJ571459, [c]FJ619254	+
	Hypocrea atroviridis	OY4107	FJ619243, [c]FJ619251	+
	Hypocreales	OY360	FJ571460	–
	Paraphaeosphaeria sp.	OY13707	FJ571461	+
	Penicillium sp.	OY7707	FJ571462, [b]FJ619257	+
	Penicillium brevicompactum	OY12607	FJ571464, [b]FJ619258	–
	Penicillium brevicompactum	OY8407	FJ571463, [b]FJ619259	–
	Penicillium chrysogenum	OY4207	FJ571467	+
	Penicillium citrinum	OY30207	FJ571468	–
	Penicillium crustosum	OY10807	FJ571469	+
	Penicillium glabrum	OY4507	FJ571470	+

Table 2 (continued)

Phylum	Taxon	Strain no.	[1]GenBank Accession No.	Inhibitory activity
	Penicillium implicatum	OY15607	FJ571471, [b]FJ619261	+
	Penicillium pinophilum	OY16407	FJ571472, [b]FJ619262	+
	Penicillium piscarium	OY5307	FJ571474, [b]FJ619263	–
	Penicillium sp. nov. (*P. pinophilum*/*purpurogenum* complex)	OY12007	FJ571473, [b]FJ619264	+
	Penicillium sp. nov. 2 (*P. mineoluteum*/*aculeatum* complex)	OY17107	FJ571465, [b]FJ619265	–
	Penicillium sp. nov. 3 (*P. purpogenum*/*pinophilum* complex)	OY18307	FJ571475, [b]FJ619266	+
	Penicillium sp.	OY30307	FJ571466, [b]FJ619267	+
	Penicillium steckii	OY807	FJ571476, [b]FJ62937	+
	Phoma leveillei	OY13807	FJ571477	+
	Phomopsis sp.	OY363	FJ571478	–
	Plectosphaerella sp.	OY407	FJ571479	–
	Pleosporales	OY1507	FJ571480	+
	Pleosporales	OY16507	FJ571481	+
	Pleosporales	OY19507	FJ571482	–
	Preussia sp.	OY2307	FJ571483	–
	Sordariomycetes	OY36907	FJ571484	–
	Stachybotrys sp.	OY4307	FJ571485	–
	Trichoderma atroviride	OY3807	FJ571486, [c]FJ629374	–
	T. harzianum	OY1107	FJ571488, [c]FJ619253	+
	T. harzianum	OY3207	FJ571487, [c]FJ619249	+
	T. longibrachiatum	OY6607	FJ571489, [c]FJ619248	+
	Trichoderma. sp. nov. 1 (Strictipilosa clade)	OY1007	FJ571490, [c]FJ619255	+
	Trichoderma sp.	OY1607	FJ571496	+
	Trichoderma. sp. nov. 2 (Strictipilosa clade)	OY2407	FJ571491, [c]FJ619250	+
	Trichoderma sp.	OY5007	FJ571492	+
	Trichoderma. sp. nov. (Section Longibrachiatum)	OY7107	FJ571493, [c]FJ619252	+
	Trichoderma sp.	OY14707	FJ619244, [c]FJ619256	+
	Trichurus sp.	OY16707	FJ571495	–
	Verticillium sp.	OY1207	FJ571497	– (+P)
Basidiomycota	*Coprinellus* sp.	OY35307	FJ571499	+ (–P)
	Coprinellus sp.	OY9407	FJ571498	+ (–P)
	Trichosporon sp.	OY4907	FJ571500	–
Glomeromycota	Mucoraceae	OY3507	FJ571501	–
	Rhizopus sp.	OY11107	FJ571502	–

strains, a majority (94%) were identified as Ascomycota. Basidiomycota (4%) and Glomeromycota (2%) were also isolated from the sponge samples (Table 1). Within the Ascomycota (a total of 80 taxa), 25 were identified as *Eurotiales* (*Aspergillus* and *Penicillium* spp.), 5 as *Capnodiales* (e.g., *Cladosporium* spp), 15 as *Pleosporales* (*Bionectria*, *Fusarium*, *Phoma*, and *Preusia* spp.) and 35 taxa as *Hypocreales* (*Acremonium* and *Trichoderma* spp.) (Fig. 2).

To determine if inhibiting the growth of some taxa during the isolation procedure would increase the diversity of fungi obtained, sponge samples were placed on media with different fungicidal or other anti-biotic amendments. On each of the fungicide-amended PDACl (FPDA) plates we could repeatedly find fungal taxa which were found to recur on different FPDA media, as well as unique taxa that were specifically tolerant/resistant to a given fungicide (with the exception of panoctine, ardent and soprano). By using the FPDA media, we isolated 28 different taxa that were not found using the standard (unamended) medium (Fig. 3). Overall, the amendments which proved to be the most effective in diversifying the taxa obtained (in terms of recurring versus unique) were cycloheximide, benlate and bupirimate.

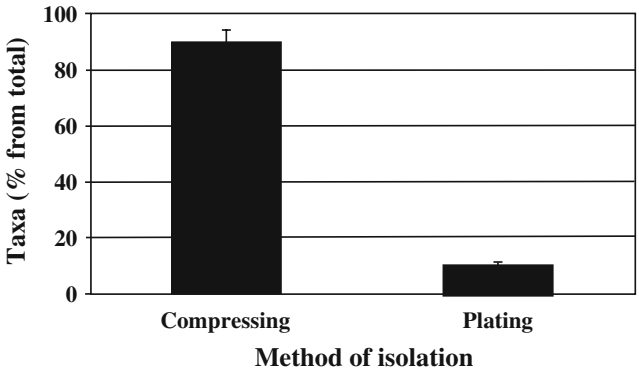

Fig. 1 Fungal taxa yield from each of the isolation methods, presented as percentage from total taxa. *Error bars* indicate standard deviation (at 5% level of confidence)

Based on the relative ease in which it was possible to isolate fungi associated with the sponge tissue and the fact that a high variety of fungi are present within the sponge it was hypothesized that these species have the potential to exhibit anti-fungal properties. This would be expected in cases where competition within the niche may occur (Bhadury et al. 2006). Thirty-six of the marine-derived taxa we isolated exhibited inhibitory activity, as determined by a diffusion bioassay which included four test organisms from various taxonomical divisions. The isolates which secreted inhibitory compounds into the growth medium were predominantly Hypocreales (*Trichoderma*, *Acremonium*, *Bionectria* or *Verticillium* spp.) and Eurotiales (*Penicillium* and *Aspergillus* spp.). Other taxa exhibiting inhibitory activity included two Pleosporales (strains OY1507 and OY16507) and one Basidiomycotina taxon (*Coprinellus* sp. strain 9407) belonging to the *Agaricales*. Other Basidiomycotina (*Coprinellus* sp. strain OY35307 and *Trichosporon* sp. strain OY4907) as well as the isolated Glomeromycota isolates (*Rhizopus* sp. strain OY11107 and a *Mucoraceae* sp. strain OY3507) did not exhibit inhibitory activity under the conditions tested (Table 1).

In addition to the contribution of secreted chemicals to the possible interaction between fungi occupying the same niche, it is conceivable that additional modes of fungal-fungal interactions may occur. As terrestrial *Trichoderma* species are known to exhibit mycoparasitic properties, we explored the possibility that some of the *Trichoderma* strains isolated from *Psammocinia* sp. could parasitize other fungi isolated from the same sponge environment. In order to asses this possibility, dual cultures of *Trichoderma* strains isolated during the study along with *Aspergillus* and *Fusarium* strains were established and the resulting interactions observed microscopically. We repeatedly observed typical coiling of *Trichoderma* on *Fusarium equiseti* (isolated from the same sponge), indicative of a mycoparasistic interaction (Fig. 4). However, the strains tested did not parasitize any of the *Psammocinia*-associated *Aspergillus* spp. examined (data not shown).

Discussion

Our understanding of the ecological roles fungi have in marine ecosystems in general and in marine sponges in particular is still vague (Hyde et al. 1998; Morisson-Gardiner 2002; Raghukumar 2008). Furthermore, the reported data concerning fungi associated with marine sponges is extremely limited. Traditional plating methods for studying fungal diversity in marine sponges, soil and plants has its limitations (Taylor et al. 2004; Hyde and Soytong 2008). In this study, we compressed the sponge samples, to increase the number of propagules detached from the inner sponge sample surfaces. This, along with combining the use of anti-fungal amendments to the medium, increased both the number as well as the diversity of fungal taxa isolated.

Most of the fungal taxa isolated in the present study are similar to those previously reported to be associated with other marine sponge species (Höller et al. 2000; Wang

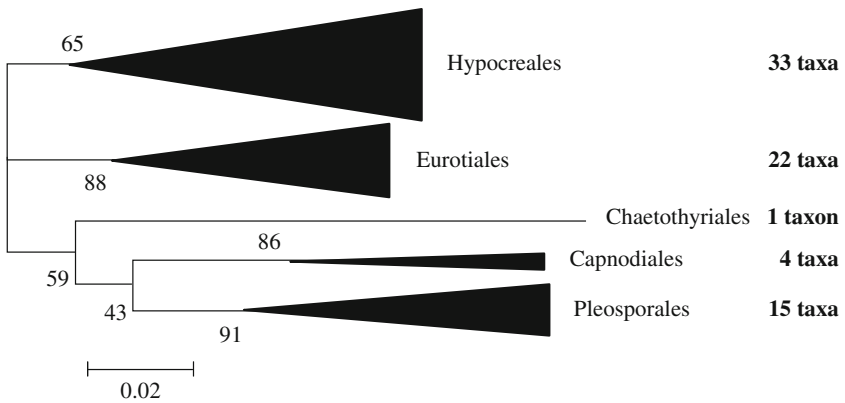

Fig. 2 Neighbor joining phylogenetic tree based on rRNA ITS sequences (>500 bp) of cultured Ascomycota fungi associated with the marine sponge *Psammocinia* sp. Number above or below branches indicate bootstrap values of neighbor-joining analysis from 1,000 replicates

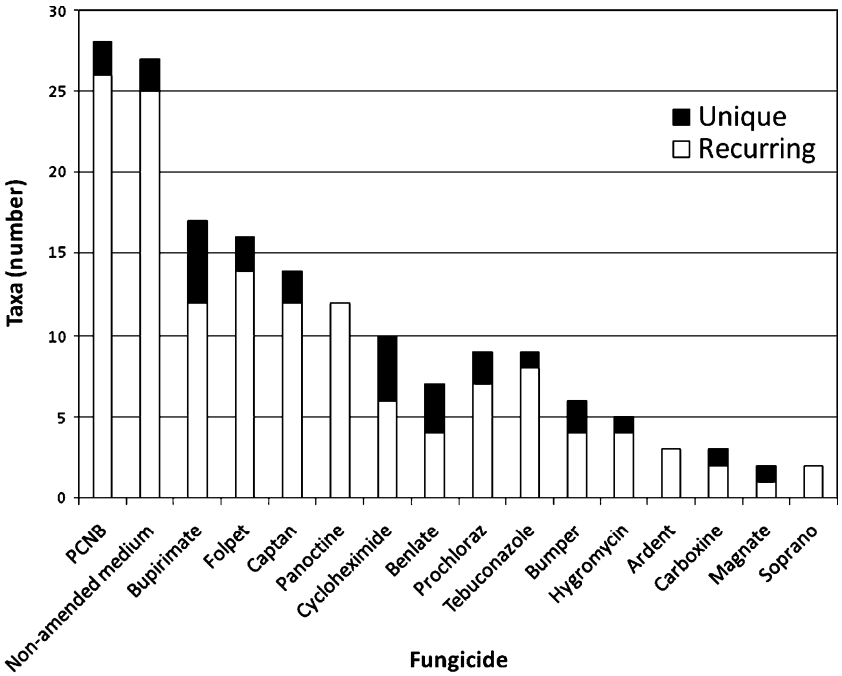

Fig. 3 The number of unique and recurring fungal taxa observed in different fungicide-amended PDACl media

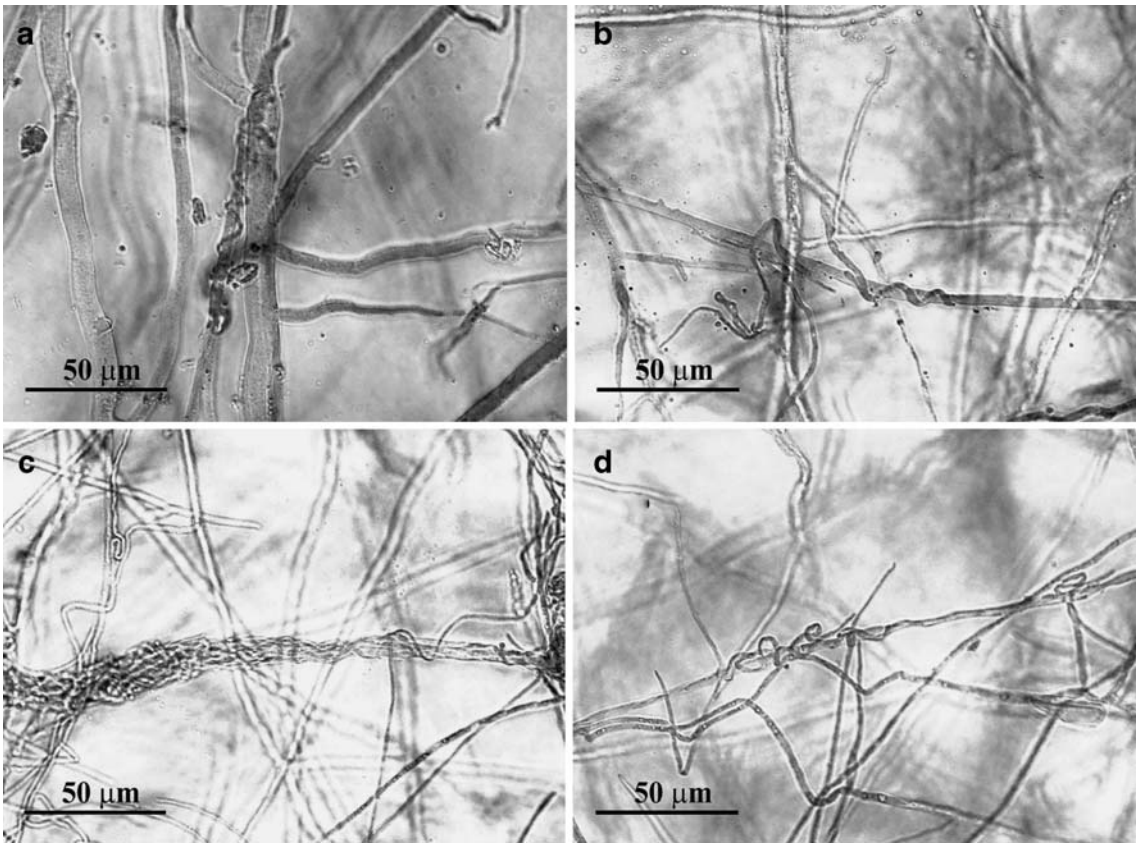

Fig 4 Coiling mycoparasitic properties of *Trichoderma* spp. (**a** *T.* sp. strain 5007); (**b** *T. longibrachiatum* strain 6607); (**c** *T. longibrachiatum* strain 7507); (**d** *T.* nov. sp. (Strictipilosa Clade strain 1007)) on *Fusarium equiseti*. All strains were isolated from *Psammocinia* sp.

2006; Wang et al. 2008). Even though identification of fungi on the basis of molecular analysis of the rRNA gene cluster is highly common (Bridge 2002), it can be subject to inaccuracies, mainly on the basis of inadequate quality control (Druzhinina et al. 2005). The results obtained here were based on linking molecular taxonomy with microscopic observations, which we believe contributed to an increase in reliability. Use of molecular techniques to identify internal fungi within plants has also resulted in more accurate identification (see Guo et al. 2001, 2003; Wang et al. 2005; Rungjindamai et al. 2008; Tao et al. 2008). We isolated 15 taxa whose molecular characterization used in this study could not provide a reliable genus-level identification, as comparable sequences in public sequence databases were lacking. Furthermore, some of the Pleosporales appear to be new to science and will be published in a follow-up paper.

The relatively high number of fungal taxa isolated in this study from a single sponge species (85 taxa), was significantly higher than in other studies that focused on assessing the quantity of marine sponge-associated fungi (Höller et al. 2000; Wang et al. 2008). The internal number of taxa isolated from plants, however has also been shown to be high (eg., Sánchez Márquez et al. 2008). This raises the question if results presented here are due to the specific niche studied, to the isolation technique used or, perhaps, are a stochastic effect related to the small number of studies performed on fungal diversity in sponges. Given the clear increase in the number of colonies obtained by using the compression method, when compared to the direct plating technique, and the fact that adding fungicidal amendments proved as efficient in isolating species that were otherwise undetected, it can be concluded that the experimental approach employed here contributed significantly to both quantitative and qualitative expansion of the fungal taxa detected. Nonetheless, it is likely that the yield of *Psammocinia*-associated fungal taxa in this study is also incomplete. One way of trying to assess *Psammocinia*-associated fungal diversity (S_{ACE}) is to employ a coverage-based estimator (Kemp and Aller 2004), using the following formula:

$$\mathbf{S}_{ACE} = \mathbf{S}_{ABUND} + \frac{\mathbf{S}_{RARE}}{\mathbf{C}_{ACE}} + \frac{\mathbf{F}_1}{\mathbf{C}_{ACE}}\gamma^2_{ACE}$$

F_1 is the number of taxa occurring once in the library, S_{RARE} is the number of taxa occurring 10 or fewer times, and S_{ABUND} is the number occurring more than 10 times in a given library. γ^2_{ACE} is the coefficient of variation of the F_i's and C_{ACE} is a sample coverage estimate. The coverage-based model, along with the online utility (www.aslo.org/lomethods/free/2004/0114a.html) used to assist us in calculating the various values suggested that in the study described here only about 15% of the fungal taxa that are associated with *Psammocinia* sp. were isolated. Even if we consider this model to be strict, it is believed that it still is far from saturation, in terms of obtaining a comprehensive *Psammocinia*-associated fungal diversity profile. It is highly likely that additional fungi, requiring alternative culturing conditions are associated with the sponge. Furthermore, the possibility of obligate sponge-dependent taxa residing in association with the sponge should not be discarded.

Bacteria associated with marine sponges may exhibit host-specificity (Taylor et al. 2004) even though being both a potential major food source along with being a potential symbiont may appear paradoxical (Hentschel et al. 2006; Taylor et al. 2007a). There is a real lack of data regarding sponge associated fungi (Höller et al. 2000) and of their possible ecological role (Gao et al. 2008; Wang et al. 2008). It is even more pronounced among Irciniid-associated microorganisms where only few studies have examined ecological aspects of such sponges and their rich microbial communities (e.g. Maldonado and Young 1998; Thakur et al. 2004; Hentschel et al. 2006) that raises the question whether similar considerations of potential bacteria-sponge interactions (and their potential contradiction) may apply in the case of fungal-sponge associations.

Many Eurotiomycetidae, Hypocreomycetidae and Trichocomaceae isolated from either terrestrial or aquatic environments have shown to secrete anti-fungal antibiotics (Bhadury et al. 2006; Gomez-Guinan et al. 2003; Höller et al. 2000). Even though Gao et al. (2008) have recently reported, following the application of DGGE, on the presence of a few uncultured Basidiomycota detected in samples obtained from the marine sponges *Suberites zeteki* and *Mycale armata*, only 12 Basidiomycota species are known that have been identified from marine environments (Jones et al. 2009). To the best of our knowledge this study presents, for the first time, three basidiomycete fungal taxa isolated from *Psammocinia* sp. (and from marine sponges), two of which exhibit in vitro anti-fungal activity.

In this study, we demonstrated the ability of different *Trichoderma* spp. isolated from *Psammocinia* sp. samples to mycoparasite *Fusarium equiseti* isolated from the same sponge specimen. These assays were conducted in `semi` marine conditions (sea water agar). Given the fact that evidence for the formation of fungal hyphae in any marine sponge has yet to be presented, the ability of *Trichoderma* sp. mycoparsitising other sponge-associated fungi remains a speculation.

Despite the success in isolating numerous species from a sponge it is clear that our methodology limited. By using anti-fungal agents in the media we were able to isolate a greater diversity of taxa. Studies on the internal fungi of plants (endophytes) have shown that taxa exist which were not isolated using culture-based techniques (Hyde and Soytong

2008). Future studies on internal sponge fungal diversity should therefore combine molecular techniques for direct evaluation of fungal diversity using total DNA extracted from sponges (Duong et al. 2006; Seena et al. 2008).

In summary, we have been successful in isolating a range of fungal taxa using sponge sample compression and different media amended with fungicides. Many of the isolated fungal strains can secrete anti-fungal compounds. The study however begs the question concerning the role of sponge-associated fungi within the host organism. *Aspergillus sydowii* is a pathogen of soft coral in the Caribbean (Geiser et al. 1998), yet was isolated from a sponge that was apparently healthy (Ein-Gil et al. 2009), as were the fungi isolated in this study. Since sponges are filter feeders; it is conceivable that spores of fungi have been filtered from the water and are lodged in the sponge tissues, thus having no active role in the biology of the sponge. Nonetheless, as fungal propagules can survive for significant periods within some sponges, a live sponge may be a symptomless carrier of a pathogen of other marine biota. However, whether or not substantial fungal (pathogen or other) growth and dissemination occur in living sponges, has yet to be determined (Ein-Gil et al. 2009). The types of taxa we isolated here are not usually found on substrates submerged in the sea (Jones et al. 2009). The nature of these taxa and whether the compounds they produce are secreted within the sponge, and whether or not they play a role in the chemical ecology of the sponge has yet to be elucidated.

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