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Diversity of cultivable fungi associated with Antarctic marine sponges and screening for their antimicrobial, antitumoral and antioxidant potential

Marlene Henríquez · Karen Vergara · Javiera Norambuena · Andrea Beiza ·
Felipe Maza · Pamela Ubilla · Ivanna Araya · Renato Chávez · Aurelio San-Martín ·
José Darias · María J. Darias · Inmaculada Vaca

Abstract The diversity of sponge-associated fungi has been poorly investigated in remote geographical areas like Antarctica. In this study, 101 phenotypically different fungal isolates were obtained from 11 sponge samples collected in King George Island, Antarctica. The analysis of ITS sequences revealed that they belong to the phylum Ascomycota. Sixty-five isolates belong to the genera *Geomyces*, *Penicillium*, *Epicoccum*, *Pseudeurotium*, *Thelebolus*, *Cladosporium, Aspergillus*, *Aureobasidium, Phoma*, and *Trichocladium* but 36 isolates could not be identified at genus level. In order to estimate the potential of these isolates as producers of interesting bioactivities, antimicrobial, antitumoral and antioxidant activities of fungal culture extracts were assayed. Around 51 % of the extracts, mainly from the genus *Geomyces* and non identified relatives, showed antimicrobial activity against some of the bacteria tested. On the other hand, around 42 % of the extracts showed potent antitumoral activity, *Geomyces* sp. having the best performance. Finally, the potential of the isolated fungi as producers of antioxidant activity seems to be moderate. Our results suggest that fungi associated with Antarctic sponges, particularly *Geomyces*, would be valuable sources of antimicrobial and antitumoral compounds. To our knowledge, this is the first report describing the biodiversity and the metabolic potential of fungi associated with Antarctic marine sponges.

Keywords Antarctic sponges · Fungi · *Geomyces* sp. · Antimicrobial · Antitumoral · Antioxidant

M. Henríquez · K. Vergara · J. Norambuena · A. Beiza ·
F. Maza · I. Araya · A. San-Martín · I. Vaca (✉)
Departamento de Química, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile
e-mail: inmavaca@uchile.cl

P. Ubilla · R. Chávez
Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile (USACH), Alameda 3363, 9170022 Estación Central, Santiago, Chile

J. Darias
Instituto de Productos Naturales y Agrobiología (CSIC), Avenida Astrofísico F. Sánchez, 3, 28206 La Laguna, Tenerife, Spain

M. J. Darias
IRTA, Centre de Sant Carles de la Rapita, 43540 Sant Carles de la Ràpita, Spain

M. J. Darias
IRD, UMR226-I-SEM (Institut de Sciences de l'Evolution de Montpellier), 34196 Montpellier Cedex 5, France

Introduction

With more than 70 % of the planet's surface covered by water, oceans are probably the most promising habitat to explore for novel chemicals with biological properties. However, currently is recognized that just a little fraction of these chemicals from marine sources have been studied and identified. In this context, sponges (phylum Porifera) have been described as a rich source of bioactive compounds with biotechnological interest, such as antimicrobial, antitumoral, and others (Wang 2006; Blunt et al. 2007).

One interesting aspect of sponges is that these organisms maintain symbiotic relationships with microorganisms. Sponges are commonly known to harbor diverse microbes and in fact, they can compose up to 50 % of the sponge tissue volume exceeding microorganisms in seawater by 2–4 orders of magnitude (Hentschel et al. 2006). Many compounds from marine macroorganisms bear structural resemblance to those from microbes (for examples see

Moore 1999; Schmidt et al. 2000), suggesting that most of the biological activities from marine sponges could be truly produced by the microbiota associated. This fact has drawn the attention of researchers to the study of biological activities produced by sponges-associated microbes, which has led to the obtainment of several clinically important bioactive compounds from these microbes (Thomas et al. 2010).

The study about the diversity of the sponge-associated microbes is still fragmentary. In contrast to prokaryotic microbes associated with sponges, studies about the diversity of sponge-associated fungi are still scarce (Zhou et al. 2011). Fungi associated with sponges have been described mainly in sponges from temperate shallow-water, tropical, and Mediterranean systems, particularly in the Atlantic Ocean (Baker et al. 2009; Menezes et al. 2010), the Mediterranean Sea (Wiese et al. 2011; Paz et al. 2010), the Indian Ocean (Thirunavukkarasu et al. 2012), the South China Sea (Zhou et al. 2011; Liu et al. 2010; Ding et al. 2011) and the Pacific Ocean (Wang et al. 2008; Gao et al. 2008; Li and Wang 2009). On the contrary, studies about sponge-associated fungi from cold marine environments, such as Antarctic seas, are extremely scarce. In many Antarctic benthic communities, sponges are one of the dominant macroinvertebrate organisms (Avila et al. 2007), and the presence of bacteria, archaea and benthic diatoms has been reported in them (Webster et al. 2004). However, to the best of our knowledge, the presence of fungi associated with Antarctic sponges has remained virtually ignored.

Numerous studies have showed that sponge-associated fungi are one of the major marine sources of bioactive compounds (Bugni and Ireland 2004; Raghukumar 2008). Due to the increasing need of new antibiotic drugs with novel mechanisms of action for which bacteria have not developed still resistances, antimicrobial activity is one of the most searched properties in sponge-associated fungi (Zhou et al. 2011; Baker et al. 2009; Liu et al. 2010; Ding et al. 2011). The results of these assays have shown that fungi isolated from marine sponges are good sources of new antibiotics. On the contrary, other bioactivities such as antitumoral and antioxidant have been less studied in sponge-associated fungi. Compounds with antitumoral activity have been described in few fungi associated with sponges (Thomas et al. 2010; Liu et al. 2010). On the hand, a recent study suggests that sponge-associated fungi could be good producers of antioxidant compounds (Thirunavukkarasu et al. 2012), but as antitumoral, this is a poorly explored activity in these organisms.

Taking in account that diversity of fungi associated with Antarctic marine sponges and their potential as producers of interesting bioactivities have been poorly studied, our aim was to study, for the first time, the diversity of cultivable fungi associated with Antarctic sponges, and to describe the potential of these isolated fungi as producers of antimicrobial, antitumoral and antioxidant activities.

Materials and methods

Sponge collection

Fragments of 10–15 cm of eleven marine sponges belonging to genera *Dendrilla* sp. (two specimens), *Tedania* sp. (three specimens), *Hymeniacidon* sp. (three specimens), and 3 unidentified sponges from the order *Poecilosclerida* (probably from genera *Microciona* (*Clathria*) sp. and/or *Crella* sp.), were collected using scuba diving in Fildes Bay (62°12′0″S 58°57′51″W), King George Island (Antarctica) at a depth of 6 m. Once excised, sponge specimens were transferred directly to sterilized zip-lock bags containing seawater to prevent contact of sponge tissue with air, and cooled on ice. The samples were transported to the laboratory facilities in Professor Julio Escudero Base located by Fildes Bay and processed immediately for fungal isolation.

Isolation of sponge-associated fungi

Sponges were rinsed with sterile solution 0.9 % NaCl. After that, two parallel procedures for the processing of the sponges were used. First, pieces of approximately 1 cm³ of the inner tissues from each sponge were excised under sterile conditions with a scalpel and forceps, and directly spread onto Petri plates containing different culture media (see below). Alternatively, other samples of the inner tissues were homogenized with a minimal volume of 0.9 % NaCl under aseptic conditions by a high-speed pulse-type homogenizer machine. One hundred microliters of each homogenate were spread onto Petri plates as mentioned above.

Culture media used were potato dextrose agar (PDA, Difco), PDAMM (PDA plus 2 g/l NaCl), GPY (1 g/l glucose, 0.1 g/l yeast extract, 0.5 g/l peptone, 15 g/l agar), and GPYMM (GPY plus 3 g/l NaCl), all of them containing the antibiotics benzylpenicillin and streptomycin (100 μg/ml each) to prevent bacterial growth. The plates were incubated at 20 °C for 1–3 weeks. Each fungal isolate obtained was individually picked and transferred onto a new PDA plates containing antibiotics as above and incubated at 20 °C by 12 days. In order to obtain pure axenic cultures, each fungal colony obtained was submitted to serial dilutions (1:1,000, 1:10,000, and 1:100,000) in 0.9 % NaCl and subsequently, 100 μl of each dilution were plated in duplicate onto PDA plates. Plates were incubated as above, colonies obtained were picked, grown onto a new PDA plates, and submitted to serial dilution again. This procedure was repeated until the morphology of all the colonies of the dilutions was undistinguishable.

Molecular identification and phylogenetic analysis of cultivated fungi

All fungi were grown on the following media: CYA, PDA, NO2, CREA, MEA, and Cz-Dox (Frisvad and Samson 2004). Phenotypical traits (morphology and colour of mycelia and conidia) were examined to exclude redundant fungal isolates.

For the extraction of fungal DNA, the fungal isolates were grown in CYA broth at 20 °C and 200 rev/min for 2–4 days. The mycelium was harvested using Nytal filter, washed with solution 0.9 % NaCl, and dried between two layers of paper towel. The resulting mycelial mat was ground using liquid nitrogen. Fungal DNA was extracted using the procedure described by Navarrete et al. (2009). In brief, the mycelial powder was transferred to a 2 ml tube containing 800 µl of Grinding buffer (0.2 M Tris, 10 mM EDTA, 1 % SDS, pH 8.2) and 800 µl of phenol solution. After a brief mixing, the mixture was heated at 50 °C for 30 min. After centrifugation at 14,000 rev/min for 10 min, the aqueous phase was transferred to a new microtube, extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1). Finally, DNA was precipitated by adding two volumes of ethanol and 1/10 volumes of acetate of sodium 3 M pH 5.2. The DNA pellet was washed with 70 % ethanol and re-suspended in 50 µl of sterile water. Each DNA was used as template to amplify the fungal ITS-rDNA fragments using the primers ITS1 (5′-TCCGTA GGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′) (White et al. 1990). PCR conditions were as follows: initial denaturation (94 °C for 5 min), 30 cycles of denaturation (94 °C for 50 s), primer annealing (55 °C for 50 s), and elongation (72 °C for 1 min), with a final elongation at 72 °C for 1 °C min. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and used directly for sequencing analysis (Macrogen Inc., Korea).

The sequences obtained were submitted to the BlastN, matching with GenBank database sequences. Multiple alignments were carried out using ClustalX. Data from ClustalX were exported to the Mega 5 package to build phylogenetic trees using the maximum-parsimony method, using close-neighbor-interchange algorithm (search level = 2, random additions = 100). The quality of the consensus tree was examined by bootstrap re-sampling of the data sets with 1,000 replications.

Antimicrobial, antitumoral, and antioxidant assays of isolated fungi

Fungal isolates were cultured in 500 ml Erlenmeyer flasks containing 100 ml of CYA medium. After incubation for 10–12 days at 20 °C and 200 rev/min, the mycelium was separated from the culture medium by filtration. The fermentation broth was extracted with ethyl acetate (3 × 60 ml). This procedure is widely used to extract compounds from fungal culture broths (Wiese et al. 2011). Then, the filtrate was adjusted to pH 2.0 with 1 N HCl and extracted again with ethyl acetate. Finally, the organic phase was dried under vacuum using a rotary evaporator to give a solid or oily extract, which was used to perform bioassays (see below). Since the aqueous fraction resulting from the extraction with ethyl acetate lacks bioactivities (data not shown), this fraction was discarded.

The antimicrobial assay was carried out by agar diffusion assay. Briefly, paper discs were impregnated with 1 mg of crude extracts dissolved in 10 µl of methanol. The methanol was allowed to evaporate and the discs were placed upon agar plates containing bacterial strains *Pseudomonas aeruginosa*, *Staphylococcus aureus* ATCC25922, *Clavibacter michiganensis* 807 and *Xanthomonas campestris* 833. The plates were incubated at 37 °C (*P. aeruginosa* and *S. aureus*) or at 27 °C (*C. michiganensis* and *X. campestris*). After 24 h of incubation, inhibition zones were measured. Negative controls were only treated with methanol; positive controls were treated with benzylpenicillin (Calbiochem) and streptomycin (Sigma-Aldrich).

For antitumoral activity, the procedure describes by McLaughlin and Rogers (1998) was followed. Previous to use, potatoes were sterilized by immersing in 1 % NaOCl for 30 min. After this, potato discs (5 mm × 8 mm) were made with cork borer and placed on agar (2 %) plates (5 discs per plate). On the other hand, *Agrobacterium tumefaciens* At348 was grown for 48 h in Luria broth medium, and used to prepare each inoculation mix. Each inoculation mix was prepared by mixing 2 ml of *Agrobacterium* culture, 1.5 ml of water and 0.5 ml of the corresponding test extract solution (2 mg of extract in 0.5 ml of DMSO). Then, the surface of each potato disc was inoculated with 50 µl of each inoculation mix. Control discs were prepared in the same way, except that the test extracts were omitted. Camptothecin served as a positive control of inhibition. Fifteen potato discs were used for each test and for the control. Potato discs were incubated at 28 °C for 21 days. After staining the discs with Lugol solution (10 % KI and 5 % I_2) for 30 min, the number of tumors per disc was counted and the percent inhibition for each extract was determined using the following formula:

$$\% \text{ inhibition} = 100 - (\text{average no of tumors of sample}/ \text{average no of tumors of control}) \times 100$$

To estimate the antioxidant capacity of fungal extracts, the 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method was followed (Liu and Nair 2010). This

method has proved to be simple, rapid, inexpensive, sensitive and accurate (Muraina et al. 2009). In brief, extracts were dissolved in DMSO (4 mg/ml) and MTT was dissolved in water (1 mg/ml). An aliquot of 570 μl of MTT solution, and 30 μl of dissolved extract were vortexed in a capped glass vial for 1 min. Then, 600 μl of DMSO were added and the solution was vortexed again. The reaction was incubated at 25 °C overnight, and the absorbance was measure at 570 nm. Each sample was assayed in triplicate. Stock solutions (1 mg/ml) of control compounds (lauric acid as negative control and ascorbic acid as positive control) were also used. The antioxidant capacity was determined by comparing the absorbance of ascorbic acid (ODa) with absorbance of each extract (ODe) according to equation:

$$\% \text{ antioxidant capacity} = (ODa/ODe) \times 100.$$

Nucleotide sequence accession numbers

Fungal rDNA-ITS sequences obtained in this study were deposited in GenBank under accession numbers JX845279–JX845302.

Results

Phylogenetic diversity of cultivable fungi associated with Antarctic sponges

In total, 101 phenotypically different strains were obtained from 11 sponges samples collected in Fildes Bay, Antarctica, which were submitted to DNA extraction and sequence analysis of the ITS1-5.8S-ITS2 region. Table 1 summarizes these results. Interestingly, and although the phenotypic differences among the 101 strains were clearly notorious, only 24 distinctive ITS genotypes were detected (Table 1), indicating that despite the diverse phenotypes observed, several isolates belong to the same fungal species. The sequence analysis revealed that all the fungal isolates belong to the phylum Ascomycota, and they are affiliated to four taxonomic classes (Fig. 1): Leotiomycetes (76 isolates; 12 ITS types), Dothideomycetes (13 isolates; 5 ITS types), Eurotiomycetes (8 isolates; 5 ITS types) and Sordiaromycetes (4 isolates; 2 ITS types).

As shown in Table 1 and the phylogenetic tree (Fig. 1), most of fungal isolates obtained in the class Leotiomycetes belong to genus *Geomyces* (33 isolates, 32.7 %). Eleven isolates were identified as *Geomyces pannorum* (Table 1). Interestingly, the other 22 *Geomyces* isolates could not be identified to species level by comparing their ITS sequences with the databases, and they were closely affiliated (99–100 % identity) with *Geomyces* sp. (Table 1). Other isolates into the class Leotiomycetes were highly related

with an uncultured *Pseudeurotium* from Himalaya (Gao and Yang 2010) and to *Thelebolus* sp. from Antarctic soils (Table 1). It is noteworthy that most of the fungi in class Leotiomycetes (33 isolates, 32.7 %) could not be identified to genus level by comparison of their ITS with sequences from database. In this group, collectively named Fungal sp., the ITS sequences were grouped into five ITS types, which were highly related (99–100 %) with unidentified isolates from Antarctic soils (Table 1, Fig. 1). According to their phylogenetic placement (Fig. 1), we think that these isolates probably belong to non identified *Geomyces* spp. or closer relatives. The formal taxonomy of these isolates will be necessary to confirm this suggestion. Other three non identified isolates are described below.

Class Dothideomycetes includes orders *Dothiteales*, *Tuberculariales*, *Pleosporales* and *Capnodiales* which are represented by *Aureobasidium pullulans* (1 isolate, 1 %), *Epicoccum* sp. (6 isolates, 5.9 %), *Phoma herbarum* (1 isolate, 1 %) and *Cladosporium* spp. (5 isolates, 4.9 %), respectively (Fig. 1, Table 1).

Fungal isolates belonging to class Eurotiomycetes were closely affiliated with 2 genera of the order *Eurotiales*: *Penicillium* (7 isolates, 6.9 %) and *Aspergillus* (1 isolate, 1 %). Two isolates were identified as *P. polonicum* and five were highly related (99–100 % identity) to unidentified *Penicillium* species from Russia and Antarctica (Table 1). Finally, one isolate was identified as *Aspergillus versicolor*. The closest relatives of the isolates of *Penicillium* and *Aspergillus* obtained by us are strains from cold climates (Table 1).

Finally, four isolates were affiliated to class Sordariomycetes. One of these isolates was highly related (99 % identity) with *Trichocladium* sp. (order *Sordariales*) isolated from bat hibernaculum (Table 1). On the other hand, three isolates with identical ITS (ITS type 23) were distantly related to an unidentified isolate from air sample (94 % identity; Table 1). Analyzing the rest of the BLAST report obtained with this ITS, several sequences from *Acremonium* spp., showing low similarity with ITS type 23, were also observed. Thus, this result suggests that these three isolates belong to the class Sordariomycetes. The phylogenetic placement obtained supports this suggestion (Fig. 1), being these isolates, probably, a non-identified *Acremonium* species or a closer relative.

Antimicrobial, antitumoral and antioxidant activity of sponge-associated cultivable fungi

From the 101 fungal extracts assayed, 52 extracts (51 %) showed antibacterial activity against, at least, one of the bacteria tested (Table 1). In general, fungal extracts were most active against Gram positive (particularly against *Staphylococcus aureus*) than Gram negative bacteria

Table 1 Phylogenetic affiliations, antimicrobial, antitumoral and antioxidant activities of cultivable fungi associated with Antarctic sponges

ITS type	Closest relative (GenBank accession no)	Identity (%)[a]	Origin of the relative	Isolate	Antimicrobial activity	Crown gall tumor inhibition (%)	Antioxidant activity (%)[b]
ITS type 1	*Geomyces pannorum* (GU212398)	100	Antarctic soil and wooden structures of historic sites in the Antarctic Peninsula	F09-T1-1	–	46.1 ± 10.1	1.4 ± 0.7
				F09-T1-2	–	22.4 ± 6.9	14.9 ± 3.3
				F09-T1-3	Sa (+), Xc(++)	72.8 ± 8.7	4.2 ± 1.1
				F09-T1-4	–	88.3 ± 6.8	4.1 ± 1.7
				F09-T1-5	–	49.0 ± 10.3	2.4 ± 1.0
				F09-T1-6	Sa (+), Xc(++)	75.2 ± 12.1	11.7 ± 6.9
				F09-T1-7	Sa (+)	83.3 ± 13.3	15.2 ± 0.7
				F09-T1-8	Pa (+), Sa (+), Cm (+), Xc (++)	−68.6 ± 13.5	6.7 ± 2.6
				F09-T1-9	Cm (+)	93.3 ± 11.5	0.8 ± 1.4
				F09-T1-10	–	78.4 ± 19.5	0 ± 0
ITS type 2	*Geomyces pannorum* (DQ189227)	99	Arctic marine deposits	F09-T2-1	Sa (+++)	100 ± 0	2.2 ± 1.6
ITS type 3	*Geomyces* sp. (JN630629)	100	Antarctic soils	F09-T3-1	–	49.6 ± 15.4	8.9 ± 2.7
				F09-T3-2	Cm (+)	−29.5 ± 9.9	7.1 ± 1.9
				F09-T3-3	–	63.2 ± 10.5	11.8 ± 2.5
				F09-T3-4	–	−63.3 ± 15.3	3.1 ± 1.2
				F09-T3-5	Pa (+), Sa (+), Xc (+)	51.9 ± 14.8	2.7 ± 1.2
				F09-T3-6	–	−19.5 ± 9.0	25.3 ± 6.2
				F09-T3-7	Pa (+), Sa (+), Cm (+)	36.3 ± 9.8	6.7 ± 0.6
				F09-T3-8	–	−25.7 ± 9.2	0 ± 0
				F09-T3-9	–	15.0 ± 5.4	6.3 ± 0.4
				F09-T3-10	Sa (+), Xc (++)	81.6 ± 13.4	3.9 ± 0.5
				F09-T3-11	Sa (+), Xc (++)	88.5 ± 6.9	3.7 ± 0.3
				F09-T3-12	–	−74.8 ± 0.9	3.5 ± 1.3
				F09-T3-13	Sa (+)	48.7 ± 12.3	10.0 ± 2.6
				F09-T3-14	–	68.3 ± 8.5	2.5 ± 1.1
				F09-T3-15	Cm (+)	100 ± 0	2.9 ± 1.7
				F09-T3-16	Cm (+)	85.9 ± 12.2	30.5 ± 4.6
				F09-T3-17	Sa (+), Xc (++)	53.6 ± 17.0	7.5 ± 1.0
				F09-T3-18	–	−64.4 ± 12.6	1.7 ± 1.1
				F09-T3-19	Pa (+), Sa (+), Cm (+), Xc (++)	15.5 ± 3.1	3.1 ± 1.0
				F09-T3-20	–	10.3 ± 1.1	6.3 ± 1.7
				F09-T3-21	Sa (+)	100 ± 0	5.4 ± 1.9
ITS type 4	*Geomyces* sp. (JN630629)	99	Antarctic soils	F09-T4-1	Sa (+)	41.0 ± 7.8	5.0 ± 1.1
ITS type 5	Uncultured *Pseudeurotium* (FJ378726)	99	Soil collected in Himalaya	F09-T5-1	–	82.8 ± 4.7	1.4 ± 1.2
				F09-T5-2	–	−16.1 ± 8.0	11.6 ± 3.5
				F09-T5-3	–	19.7 ± 13.3	0.4 ± 0.2
				F09-T5-4	Sa (++)	−31.7 ± 13.3	7.0 ± 1.5
ITS type 6	Uncultured *Pseudeurotium* (FJ378726)	99	Soil collected in Himalaya	F09-T6-1	–	34.8 ± 11.3	4.5 ± 0.4
ITS type 7	*Penicillium polonicum* (HQ850925)	99	Non determined	F09-T7-1	Sa (++)	−81.3 ± 0.8	36.8 ± 12.4
				F09-T7-2	Sa (++), Cm (++), Xc (+)	75.7 ± 7.1	2.1 ± 0.9
ITS type 8	*Penicillium commune* (JN831239)	100	Non specified Antarctic environment	F09-T8-1	Sa (+), Xc (+)	88.3 ± 10.8	19.9 ± 4.3
				F09-T8-2	Sa (+++)	−82.5 ± 9.0	11.8 ± 3.5
ITS type 9	*Penicillium solitum* (JN642222)	100	Non determined	F09-T9-1	–	33.6 ± 10.2	0.9 ± 0.8
				F09-T9-2	–	7.9 ± 4.5	1.1 ± 1.0
ITS type 10	*Penicillium commune* (JN831239)	99	Non specified Antarctic environment	F09-T10-1	Sa (++), Cm (++)	48.3 ± 14.2	36.4 ± 7.1

Table 1 continued

ITS type	Closest relative (GenBank accession no)	Identity (%)[a]	Origin of the relative	Isolate	Antimicrobial activity	Crown gall tumor inhibition (%)	Antioxidant activity (%)[b]
ITS type 11	*Aspergillus versicolor* (JN689347)	100	Endophytic fungus isolated from grass collected in Canada	F09-T11-1	–	90.5 ± 13.5	4.4 ± 2.0
ITS type 12	*Cladosporium cladosporioides* (JN565298)	100	Grapes from China	F09-T12-1	–	50.7 ± 11.9	2.2 ± 0.8
				F09-T12-2	Sa (++)	38.5 ± 5.7	1.9 ± 1.7
ITS type 13	*Cladosporium* sp. (JF773595)	100	Endophyte strain from Mexico	F09-T13-1	–	−9.0 ± 8.6	0.5 ± 0.8
				F09-T13-2	Sa (+)	93.6 ± 11.0	3.1 ± 0.3
				F09-T13-3	Sa (+), Xc (++)	−84.3 ± 15.5	10.1 ± 2.7
ITS type 14	*Thelebolus* sp. (GU004201)	100	Antarctic soil	F09-T14-1	–	−538.9 ± 17.8	3.7 ± 1.8
				F09-T14-2	–	32.3 ± 8.5	33.3 ± 1.9
				F09-T14-3	Sa (++)	−49.2 ± 9.0	4.1 ± 0.3
				F09-T14-4	Sa (++)	98.4 ± 2.7	6.2 ± 1.4
				F09-T14-5	–	80.9 ± 17.2	22.2 ± 4.5
ITS type 15	*Epicoccum* sp. (JF773624)	100	Endophyte strain from *Taxus globosa* (Mexico)	F09-T15-1	Pa (+), Sa (+)	29.4 ± 15.5	7.9 ± 0.3
				F09-T15-2	Sa (+)	25.4 ± 9.2	6.6 ± 3.6
				F09-T15-3	Sa (++)	24.5 ± 9.6	55.7 ± 3.9
				F09-T15-4	Sa (++), Xc (+)	−18.5 ± 3.7	27.4 ± 2.4
				F09-T15-5	–	−79.6 ± 10.8	3.0 ± 2.8
				F09-T15-6	Xc (+)	−59.2 ± 14.0	76.1 ± 1.5
ITS type 16	*Phoma herbarum* (FN868459)	98	Endophyte strain of *Pinus halepensis* (Spain)	F09-T16-1	–	12.7 ± 2.2	14.8 ± 2.2
ITS type 17	*Aureobasidium pullulans* (AB693902)	100	Endophyte strain from apple leaf from Japan	F09-T17-1	–	91.4 ± 9.7	22.4 ± 2.2
ITS type 18	*Fungal* sp. (GU004207)	100	Antarctic soil	F09-T18-1	Sa (++), Cm (++), Xc (++)	22.1 ± 4.5	18.6 ± 4.0
				F09-T18-2	Sa (+)	86.6 ± 8.6	1.4 ± 0.9
				F09-T18-3	–	−164.3 ± 3.4	17.3 ± 2.2
				F09-T18-4	–	91.6 ± 12.0	39.1 ± 6.1
				F09-T18-5	Sa (+), Xc (+)	−52.5 ± 5.3	9.4 ± 1.3
				F09-T18-6	Sa (++), Xc (+)	95.2 ± 6.7	5.3 ± 2.1
				F09-T18-7	–	14.9 ± 3.5	5.2 ± 1.7
				F09-T18-8	Sa (+)	−7.1 ± 3.7	3.5 ± 1.3
				F09-T18-9	–	70.0 ± 15.8	14.8 ± 3.4
				F09-T18-10	Sa (+), Xc (+)	84.6 ± 15.2	5.3 ± 0.1
				F09-T18-11	–	70.2 ± 13.2	2.6 ± 1.1
				F09-T18-12	Xc (++)	−15.5 ± 1.1	7.8 ± 0.9
				F09-T18-13	Sa (+)	51.5 ± 11.4	3.4 ± 0.7
				F09-T18-14	–	48.0 ± 10.2	4.2 ± 1.7
				F09-T18-15	Xc (+++)	−5.4 ± 6.1	4.8 ± 0.9
				F09-T18-16	–	20.7 ± 9.4	69.9 ± 5.3
				F09-T18-17	–	21.7 ± 7.1	0 ± 0
				F09-T18-18	–	−34.7 ± 10.6	3.4 ± 1.6
				F09-T18-19	Sa (+), Xc (+)	33.1 ± 9.3	0.8 ± 0.4
				F09-T18-20	Sa (+)	88.4 ± 8.6	2.0 ± 0.9
				F09-T18-21	–	−25.7 ± 13.9	15.2 ± 4.0
				F09-T18-22	Sa (++)	79.6 ± 11.0	13.4 ± 3.5
				F09-T18-23	Sa (+)	34.1 ± 11.4	7.8 ± 4.0
				F09-T18-24	Sa (+)	50.1 ± 12.4	4.6 ± 1.1
				F09-T18-25	–	81.9 ± 20.3	30.2 ± 6.0
				F09-T18-26	Sa (++)	−19.1 ± 0.7	13.2 ± 1.3
ITS type 19	*Fungal* sp. (GU004207)	99	Antarctic soil	F09-T19-1	Xc (+)	11.0 ± 2.9	0.8 ± 1.4
ITS type 20	*Fungal* sp. (GU004207)	99	Antarctic soil	F09-T20-1	–	92.6 ± 12.8	11.4 ± 4.0

Table 1 continued

ITS type	Closest relative (GenBank accession no)	Identity (%)[a]	Origin of the relative	Isolate	Antimicrobial activity	Crown gall tumor inhibition (%)	Antioxidant activity (%)[b]
ITS type 21	*Fungal* sp. (GU004206)	100	Antarctic soil	F09-T21-1	–	77.5 ± 27.6	12.2 ± 1.6
				F09-T21-2	–	82.8 ± 13.0	0.5 ± 0.8
				F09-T21-3	–	−34.3 ± 8.6	4.7 ± 0.6
				F09-T21-4	–	55.0 ± 10.2	7.0 ± 3.9
ITS type 22	*Fungal* sp. (GU004206)	99	Antarctic soil	F09-T22-1	–	−73.5 ± 5.6	9.1 ± 3.2
ITS type 23	Uncultured fungus (GQ999410)	94	Air sample from non specified environment	F09-T23-1	–	−53.3 ± 18.9	23.8 ± 4.3
				F09-T23-2	Sa (+)	80.4 ± 12.0	0 ± 0
				F09-T23-3	–	53.7 ± 16.7	0.7 ± 0.6
ITS type 24	*Trichocladium* sp. (JX270545)	99	Soil from bat hibernaculum, USA	F09-T24-1	Sa (+++), Cm (++), Xc (+)	93.3 ± 11.5	3.5 ± 1.6

Sa S. aureus, *Pa* P. aeruginosa, *Xc* X. campestris, *Cm* C. michiganensis

+++ Large inhibition zone (d > 1 cm)

++ Middle inhibition zone (1 cm ≥ d > 0.7 cm)

+ Small inhibition zone (0.7 cm ≥ d)

– No inhibition zone

[a] For this analysis, only sequences with 100 % of coverage were considered

[b] The antioxidant capacity of the extracts was calculated referring to the antioxidant capacity of ascorbic acid (see section "Materials and methods" for details)

(Fig. 2). In particular, 5 isolates (belonging to the genera *Geomyces* and *Epicoccum*) exhibited antibacterial activity against *P. aeruginosa,* 44 isolates (belonging to all the genera found except *Aureobasidium*, *Aspergillus* and *Phoma*) showed antibacterial activity against *S. aureus*, 11 isolates (belonging to Fungal sp. and genera *Geomyces* and *Penicillium*) exhibited antibacterial activity against *C. michiganensis,* and 22 isolates (belonging to Fungal sp. and genera *Geomyces*, *Penicillium*, *Epicoccum* and *Cladosporium*) exhibited antibacterial activity against *X. campestris* (Table 1). Interestingly, several fungal isolates with the same ITS type showed different antimicrobial activity (Table 1).

On the other hand, we found that several of the extracts assayed had potent antitumoral activity. Forty-three isolates (42.6 % of total) showed >50 % of inhibition of the growth of crown gall tumors on potato. Among them, 12 isolates showed >90 %, including three isolates from the genus *Geomyces* whose extracts totally inhibit growth of crown gall tumors (100 % of inhibition; Table 1). In addition, it is interesting to note that fourteen isolates (13.9 % of total) showed >50 % of stimulation of the growth of crown gall tumors (see negative values in Table 1). In some cases, this stimulation is notorious (>500 % in *Thelebolus* sp. isolate F09-T14-1; >150 % in Fungal sp. F09-T18-3).

Finally, antioxidant assay showed that from the 101 fungal extracts assayed, four were negative for antioxidant activity. Despite that all the rest showed some antioxidant activity, most of them showed activities ranging from very low to moderate activity (Table 1), and just three isolates

showed high antioxidant activities (over 60 % of antioxidant capacity of the ascorbic acid used as control). Two of these isolates, belonging to the genus *Epicoccum* (F09-T15-3 and F09-T15-6), had high antioxidant capacity (75.9 and 61.2 %). In addition, a Fungal sp. isolate (F09-T18-16) showed 69.4 % of antioxidant capacity.

Discussion

Although sponges are very abundant in Antarctica there is, to our knowledge, only one study about the diversity of microorganisms in Antarctic sponges (Webster et al. 2004). In this study, the detection of non-cultivable eukaryotic microorganisms (including fungi) was attempted using universal eukaryotic primers. The presence of diatoms and dinoflagellates was detected, but not the presence of fungi. Thus, our data of cultivable fungi associated with Antarctic sponges provide, for the first time, a look into the fungal communities living into these Antarctic marine invertebrates.

In our work, 101 fungal isolates phenotypically different were obtained from 11 Antarctic sponges samples. Interestingly, 75.2 % of isolates belong to Leotiomycetes, 12.9 % to Dothideomycetes, 7.9 % to Eurotiomycetes and 4 % to Sordiaromycetes. Compared with the prevalence of fungal classes described in sponges from other latitudes, our results suggest that fungal community of Antarctic sponges is remarkably different. For example, in Mediterranean sponges, Dothideomycetes, Eurotiomycetes and Sordiaromycetes are prevalent (between 29 and 37 % of

Fig. 1 Maximum-parsimony phylogenetic tree of ITS1-5.8S-ITS2 rDNA sequences of fungal isolates. Bootstrap values were calculated using 1,000 replications and *Chytromyces hyalinus* was used as out-group
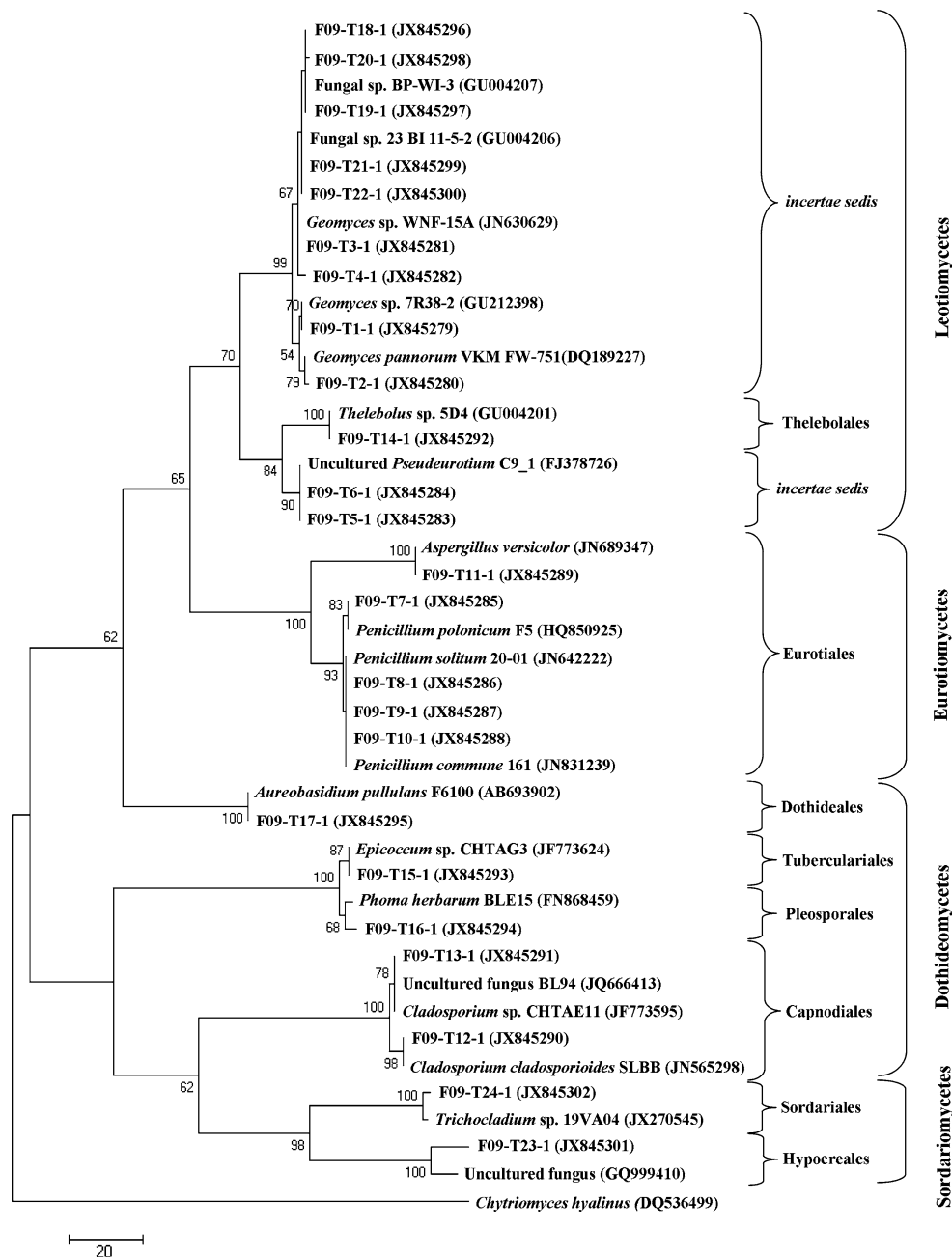
total isolates), but Leotiomycetes barely represent 1.2 % (Wiese et al. 2011). In South China Sea sponges, Eurotiomycetes (53 %) and Sordariomycetes (Hypocreales: 38.4 %) are highly prevalent, but Leotiomycetes were not found (Zhou et al. 2011). Finally, in sponges from Brazilian coasts, over 50 % of isolates belong to Sordariomycetes (mainly genus *Trichoderma*), being Eurotiomycetes and Dothideomycetes less prevalent (14 and 7 %, respectively). In this case, again, Leotiomycetes were not found (Menezes et al. 2010). Thus, and different to Antarctic marine sponges, Leotiomycetes have been rarely obtained from marine sponges. In addition, some fungal orders commonly found in sponges from other latitudes seem to be underrepresented in Antarctic marine sponges.

Although *Geomyces* spp. have been mainly described as terrestrial saprophytes that grow at cold temperatures (Blehert et al. 2009), some isolates have been obtained from cold marine environments (Loque et al. 2010; Pivkin et al. 2006). In our case, and except for an isolate closely related to an Arctic strain (Kochkina et al. 2007), all the rest of *Geomyces* isolates are relatives of *Geomyces* strains from terrestrial Antarctica (Table 1). Thus, our results suggest that in addition to soil, Antarctic *Geomyces* would be the most prevalent genus of cultivable fungi associated with Antarctic sponges.
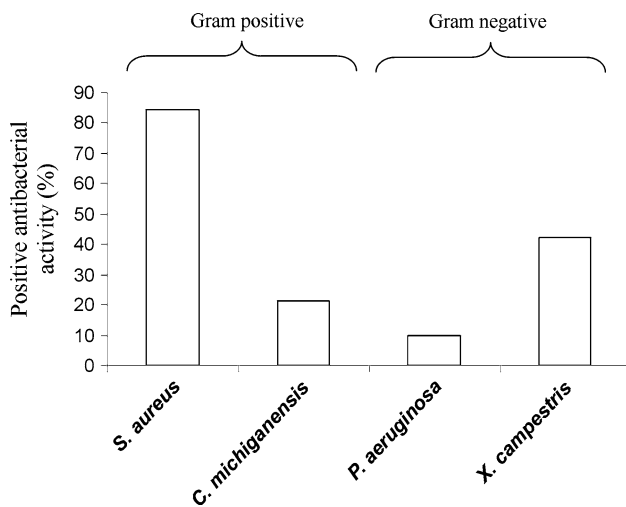
Fig. 2 The antimicrobial activities of the extracts from fungal isolates against four test microorganisms: 2 g positive and 2 g negative. For each case, one phytopathogenic and one human pathogen were chosen

Geomyces is a poorly studied genus, and in fact, the order to which this genus belongs is not clear, being classified as *Leotiomycetes incertae sedis* (*i. s*) (Fig. 1). Of the 33 isolated *Geomyces*, only eleven isolates were identified to species level as *Geomyces pannorum* (Table 1). *G. pannorum* has been previously isolated in one sponge from the Sea of Okhotsk, Russia, but its prevalence in this sponge was very low (Pivkin et al. 2006). *G. pannorum* is widely distributed in soil and non-marine aquatic habitats from terrestrial Antarctica (Bridge and Newsham 2009; Brunati et al. 2009). However, in the Antarctic marine environment, this species has been only found associated to seaweeds (Loque et al. 2010). Our results suggest that *G. pannorum* could have a broader distribution in the marine Antarctic ecosystem, including marine invertebrates. Interestingly, the other 22 *Geomyces* isolates could not be identified to species level by comparing their ITS sequences with the databases. To date, the genus *Geomyces* includes only five known species (Gargas et al. 2009). Thus, our results suggest that the number of species of *Geomyces* could be greater than heretofore described.

Other isolates into the class Leotiomycetes were highly related with an uncultured *Pseudeurotium* from Himalaya (Gao and Yang 2010) and to *Thelebolus* sp. from Antarctic soils (Table 1). As *Geomyces*, both genera have been commonly reported in terrestrial Antarctica (Bridge et al. 2009). However, to our knowledge, this is the first report of these genera in Antarctic seawater. Interestingly, *Thelebolus* sp. has been found as the predominant genus in the Antarctic lakes (de Hoog et al. 2005; Brunati et al. 2009). On the other hand, *Pseudeurotium* has been found only in sponges from the Sea of Okhotsk (Pivkin et al. 2006)

while, to our knowledge, this is the first report of *Thelebolus* sp. in sponges.

The second major group of fungi found (n = 33) includes those not identified isolates, collectively named Fungal sp., which are highly related (99–100 %) with unidentified isolates from Antarctic soils. According to their phylogenetic placement (Fig. 1), these isolates may belong to the class Leotiomycetes. Therefore, Antarctic sponges could be an important reservoir of new fungal species belong to class Leotiomycetes.

The rest of the fungi isolated from Antarctic sponges (25 isolates, 24.7 %) were found with an abundance relative of 7.9 % or less. These fungi belong to the genera *Penicillium*, *Aspergillus, Cladosporium, Epiccocum, Phoma, Aureobasidium, Trichocladium*, and *Acremonium*, which have been found in terrestrial Antarctic environments (Bridge et al. 2009). From these genera, only *Penicillium, Aureobasidium, Phoma, Trichocladium* and *Acremonium* have been reported in the Antarctic marine environment (Montemartini Corte et al. 2000; Loque et al. 2010; Grasso et al. 1997; Bridge et al. 2009). Thus, to the best of our knowledge, this work describes for the first time, the presence of *Cladosporium, Aspergillus* and *Epiccocum* in the Antarctic marine environment. The closest relatives of *Cladosporium, Epiccocum, Phoma* and *Aureobasidium* isolates obtained in this work are strains found in temperate climates (Table 1), which suggest that these isolates could be propagules introduced in the Antarctica. Conversely, the closest relatives of the isolates of *Penicillium* and *Aspergillus* obtained by us are strains from cold climates. These genera have frequently been found in soils of Antarctica suggesting that they likely come from terrestrial Antarctica. It is interesting to note that while fungi of order *Eurotiales* present high diversity in sponges from some other latitudes, including sponges from cold sea from the North Pacific (Zhou et al. 2011; Wiese et al. 2011; Pivkin et al. 2006), its abundance in Antarctic sponges is just 7.9 %.

Finally, three important bioactivities, antimicrobial, antitumoral and antioxidant, were assayed. Antimicrobial assay showed that 51 % of the fungal extracts were positive against, at least, one of the bacteria tested. In other studies where antimicrobial activity from fungi associated with sponges from other latitudes was screened, around 45 % of positive results were obtained (Ding et al. 2011; Zhou et al. 2011). Thus, our results suggest that fungi isolated from Antarctic sponges would be an important potential source of antimicrobial compounds. As was expected, since *Leotiomycetes i.s* was the most abundant fungal order obtained, its antimicrobial spectrum was more diverse. Consistently, *Geomyces* was the most prolific fungal genus, with 18 isolates (54.5 % of *Geomyces* sp.) showing antimicrobial activity (Table 1). Remarkably activity was shown by two isolates of *Geomyces* (F09-T1-8

and F09-T3-19), which were active against the four bacteria tested, suggesting that this fungal genus could have a high potential as producer of antimicrobial compounds. From the chemical point of view, the genus *Geomyces* has been poorly studied. To our knowledge, only two compounds with antimicrobial activity from *Geomyces* strains of terrestrial origin have been described (Li et al. 2008; Parish et al. 2009). Also into the class Leotiomycetes, extracts from the genus *Thelebolus* have previously shown antimicrobial activity (Brunati et al. 2009), but to our knowledge, this work is the first description of antimicrobial activity in the genus *Pseudeurotium*. Finally, most of non-identified isolates (named Fungal sp.) also were prolific as producers of antimicrobial activities, which is consistent with the fact that many of them could be Leotiomycetes (see above).

Eurotiales (*Penicillium* and *Aspergillus*) are the "classical" producers of antimicrobial activities. Accordingly, all the isolates, excepting for *P. solitum* and *A. versicolor*, produced antimicrobial activity. In the case of *P. solitum*, there are no reports in the literature concerning antibiotic production by this species (Papagianni et al. 2007), which agree with our results. However, the absence of antimicrobial activity in *A. versicolor* is somewhat surprising, since an *A. versicolor* strain previously isolated from sponges exhibited antibacterial activity against Gram positive strains, including *S. aureus* (Lee et al. 2010).

Regarding the rest of fungi, and in agreement with our results, all the Dothideomycetes and Sordariomycetes isolates obtained (*Cladosporium*, *Epicoccum*, *Phoma*, *Aureobasidium* and *Trichocladium*) have been previously described as producers of antimicrobial activities by several authors.

To determine the potential of fungi associated with Antarctic marine sponges as producers of antitumoral compounds, we used the crown gall tumor inhibition (potato disc) assay. This is a simple, inexpensive and fast screen for antitumoral activities and it has been validated by several researchers as a first general screen in the search for new antitumor compounds (Coker et al. 2003). We found that several of the extracts assayed had potent antitumoral activity (>50 % of inhibition of the growth of crown gall tumors on potato). To our knowledge, this is the first description of antitumoral activity in *Geomyces* sp. Thus, in addition to antimicrobial compounds, this fungal genus could have a high potential as producer of antitumoral compounds.

Other good producers of antitumoral activity are *A. versicolor*, *Cladosporium* sp., *Thelebolus* sp., *A. pullulans*, *Trichocladium* sp. and Fungal sp. strains (Table 1). Fungi from marine sponges producing antitumoral activities have been described before (Thomas et al. 2010), but most of them belong to other fungal genera than those described in this work. Thus, our results extend the range of fungi associated with sponges capable of producing antitumoral compounds. Interestingly, several fungal extracts stimulate growth of crown gall tumors (see negative values in Table 1). The stimulatory effect of fungal extracts on crown gall tumors has been previously observed (Nano et al. 2002), but to our knowledge, this is the first description of any proliferative activity in sponge-associated fungi. Proliferative compounds have been isolated from fungi (Teles et al. 2005), suggesting the putative presence of compounds with similar activities in sponge-associated fungi.

Finally, the antioxidant potential of the isolates seems to be limited since only thirteen isolates showed moderate antioxidant activity (between 25 and 65 % compared to the antioxidant activity of ascorbic acid). Two isolates from the genus *Epicoccum* showed good antioxidant activity (75 and 61 % of antioxidant activity of ascorbic acid). It has been described that the antioxidant properties of *Epicoccum* from marine sponges (and fungi in general) are due to the production of high quantity of extracellular polysaccharides (Sun et al. 2011). Thus, and excepting for these *Epicoccum* isolates, the potential of the antioxidant activity in fungi associated to Antarctic sponges seems to be moderate. The same conclusion was obtained by Thirunavukkarasu et al. (2012) analyzing fungi from sponges collected in Indian coast.

Taking into account the biosynthetic potential of *Geomyces* strains isolated in this work, and the poor knowledge of their chemistry, we think that Antarctic sponge-derived *Geomyces* would be good candidates for deeper studies about the production of natural products.

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