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The culturable mycobiota associated with three Atlantic sponges, including two new species: *Thelebolus balaustiformis* and *T. spongiae*

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Abstract: Covering 70 % of Earth, oceans are at the same time the most common and the environment least studied by microbiologists. Considering the large gaps in our knowledge on the presence of marine fungi in the oceans, the aim of this research was to isolate and identify the culturable fungal community within three species of sponges, namely *Dysidea fragilis*, *Pachymatisma johnstonia* and *Sycon ciliatum*, collected in the Atlantic Ocean and never studied for their associated mycobiota. Applying different isolation methods, incubation temperatures and media, and attempting to mimic the marine and sponge environments, were fundamental to increase the number of cultivable taxa. Fungi were identified using a polyphasic approach, by means of morpho-physiological, molecular and phylogenetic techniques. The sponges revealed an astonishing fungal diversity represented by 87 fungal taxa. Each sponge hosted a specific fungal community with more than half of the associated fungi being exclusive of each invertebrate. Several species isolated and identified in this work, already known in terrestrial environment, were first reported in marine ecosystems (21 species) and in association with sponges (49 species), including the two new species *Thelebolus balaustiformis* and *Thelebolus spongiae*, demonstrating that oceans are an untapped source of biodiversity.

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INTRODUCTION

Water covers almost 70 % of our planet. Nonetheless, its biodiversity and habitats remain largely unexplored. For the last 580 M years, oceans have been hosting the most ancient metazoans on Earth: sponges. *Porifera* contains more than 8 600 described species and about 15 000 species waiting to be discovered by scientists (Webster & Thomas 2016). Sponges are key components of marine ecosystems, because of their incredible ability to filter seawater: according to recent estimates, they are able to process 24 000 L/kg of seawater per day and to detain over 80 % of its particles (Taylor *et al.* 2007, Rozas *et al.* 2011).

Over the millions of years of their evolution, species of *Porifera* have formed a close association with a wide variety of microorganisms including bacteria, archaea, fungi, and algae (Taylor *et al.* 2007). This close association was described for the first time by Vacelet & Donadey (1977), who observed bacteria within sponges' tissues. Today, it is well recognised that microorganisms can represent up to 40–60 % of the sponge biomass (Yarden 2014). The term "sponge holobiont", is used when sponges and the associated microbial communities are considered as a whole (He *et al.* 2014). Different degrees of complexity characterise the interactions among sponge holobiont components, including mutualism, commensalism and parasitism (Rodríguez-Marconi 2005). Non-pathogenic microorganisms can positively contribute to sponge metabolism, by increasing the uptake of carbon, nitrogen and sulphur. Furthermore, by the production of secondary metabolites, they could be involved in host defence systems and in the regulation of the microbial community associated with sponges (Taylor *et al.* 2007). Interestingly, metabolites previously ascribed to sponges were recognised to be structurally similar to those produced by the associated bacteria (Imhoff & Stöhr 2003). As a consequence, the use of microorganisms for the bio-discovery of new molecules would avoid several problems related to the use of sponges. The isolation of new molecules and their production in the required amount from sponges is always very problematical for reasons such as their rare occurrence, difficulties with sponge collections, or irreproducible production of metabolites due to specimen variability (Imhoff & Stöhr 2003). Nowadays studies on microorganisms associated with sponges are primarily focused on prokaryotic organisms while the fungal community remain less studied, despite recent results emphasizing its great biodiversity and biotechnological potential (Raghukumar 2012). Fungi represent suitable biotechnological resources but require specific expertise for the isolation and the correct identification. Many taxa already known for their bioactivity lack a precise identification and correct preservation in culture collections hampering their possible future exploitation as recently highlighted by the 2nd International Conference of Marine Fungal Natural Products (MaFNaP 2017).

In this study we present for the first time the mycobiota associated with *Dysidea fragilis*, *Pachymatisma johnstonia* (*Demospongiae*), and *Sycon ciliatum* (*Calcarea*). The two *Demospongiae* have been extensively examined for their production of secondary metabolites. The metabolome of *D. fragilis* was characterised by Yu *et al.* (2006), although its biological activity included only a single compound, which acted as fish feeding deterrent (Marin *et al.* 1998). *Pachymatisma johnstonia* is also well known for its production of secondary metabolites, whose anticancer (Zidane *et al.* 1996, Ferreira *et al.* 2011) and antibacterial (Warabi *et al.* 2004) activity has been demonstrated. On the contrary, the metabolome of *S. ciliatum* has never been studied.

In light of the above-mentioned considerations, it is likely that microorganisms, including fungi, could be the true producers of the bioactive molecules isolated so far but could also be a source of additional novel compounds of interest. Aiming at the biotechnological exploitation of new molecules, the scope of this study was to isolate and identify the fungal community associated with three Atlantic sponges, with particular emphasis on the proper systematic identification of taxa. Moreover, in this paper two novel *Thelebolus* species are described.

MATERIAL AND METHODS

Sampling sites and axenic isolation

The sponges *Dysidea fragilis* and *Pachymatisma johnstonia* (three specimens each) were collected by scuba divers in Gurraig Sound (Co. Galway, Ireland; N 53°, 18.944; W 09°, 40.140). The sampling site was at 15 m depth, characterised by fairly strong current and suspended sediments. Three specimens of *Sycon ciliatum* exposed to a fast water flow due to the tide going out were collected in Coranroo rapids (Co. Clare, Ireland; N 053°09.100, W 009°,00. 550).

Specimens were surface sterilised with ethanol 70 % (for 30 s) to prevent contaminants and serially washed (three times) in artificial sterile Sea Water (SW) to get rid of unrefined sediments and to wash out propagules, in order to leave only fungi actively growing on the surface or into the sponge tissues.

Working in sterile conditions, the sponge samples were divided into three parts to be used for two different fungal isolation techniques and for a taxonomic voucher of the sponge. For the first isolation method, one third of the sponge was further cut in 20 pieces of about 0.5 cm^3 by means of sterile tools and directly plated in Petri dishes (six cm Ø) containing two different media: Sea Water Agar – SWA (Sea Salts 30 g, Agar 15 g, up to 1 L dH_2O) and Corn Meal Agar Sea Water - CMASW (Corn Meal 2 g, Agar 15 g, Sea Salts 30 g, up to 1 L dH_2O). Five replicates for each medium and incubation temperatures (15 °C and 25 °C) were performed.

Approximately 5 g of each sample were also homogenised (homogenizer blade Sterilmixer II - PBI International) and diluted 1:10 w/v in SW. One mL of suspension was included in Petri dishes (nine cm Ø) containing CMASW or Gelatin Agar Sea Water–GASW (Gelatin 20 g, Sea Salts 30 g, Agar 15 g, up to 1 L dH_2O), rich in collagen and mimicking sponge tissue composition. Five replicates for each medium and incubation temperatures (15 °C and 25 °C) were performed. All media were supplemented with an antibiotic mix (Gentamicin Sulfate 40 mg/L, Piperacillin plus Tazobactam 11 mg/L) to prevent bacterial growth. Plates were incubated in the dark and periodically checked for 30 d to isolate slow growing fungi.

Fungal identification

Fungal morphotypes were isolated in pure culture and identified by means of a polyphasic approach combining morpho-physiological and molecular features. After determination of genera via macroscopic and microscopic features (Domsch *et al.* 1980, Von Arx 1981, Kiffer & Morelet 1997), fungi were transferred to genus-specific media (Klich 2002, Braun *et al.* 2003, Samson & Frisvad 2004).

In parallel for molecular analyses, fungi were pre-grown on Malt Extract Agar - MEA (Malt Extract 20 g, Glucose 20 g, Peptone 2 g, Agar 20 g, up to 1 L dH_2O) at 25 °C for 1 wk, for fast growing fungi, and from 2–4 wk for slow growing fungi. DNA was extracted using the NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer instructions. Based on the taxonomic assignment attributed to each fungus by morphological observations, specific primers were used for PCR as detailed in Table 1. Briefly, PCR reactions were performed in 50 µL final volumes and consisted of 0.5 µL Taq DNA Polymerase (Qiagen 5 U/µL), 10 µL PCR Buffer (10 ×), 2.5 µL dNTP Mixture (dATP, dCTP, dGTP, dTTP; 10 mM), 2 µL $MgCl_2$ (25 mM), 2.5 µL of each primer (10 µM), 1 µL genomic DNA extract (80 ng/mL) and 34 µL distilled-deionised water. PCR products were visualised under UV light (BIO-RAD Universal Hood II) on 1.5 % agarose electrophoresis gels stained with ethidium bromide. Macrogen, Inc. (Seoul, South Korea) Europe Lab carried out the purification and sequencing of PCR products.

Taxonomic assignments were based both on high percentage homologies with sequences available in public databases (GenBank - NCBI database and CBS-KNAW Collection, Westerdijk Fungal Biodiversity Institute) and the consistency of morphological features with available literature descriptions. The taxonomic position of doubtful strains (low homologies with sequences available in public databases) or sterile mycelia (i.e. not showing morphological features useful to confirm taxonomical assignments) were inferred via molecular phylogenetic analyses based on DNA sequences from the large ribosomal subunit LSU, (Vilgalys & Hester 1990). Separate alignments were created for the orders *Pleosporales*, *Capnodiales* and *Chaetothyriales* and two for the classes *Leotiomycetes* and *Sordariomycetes*. Alignments were generated using MEGA v. 7.0 and manually refined. Phylogenetic analyses were performed using a Bayesian Inference (BI; MrBayes v. 3.2.2 four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC), run over 10 M generations, (under GTR + Γ + I evolutionary model approach). BPP values are reported in the resulting trees. A full alignment of the dataset was submitted to TreeBASE (submission number 21746).

Representative strains of each species isolated in pure culture during this work are preserved at *Mycotheca Universitatis Taurinensis* (MUT- www.mut.unito.it) of the Department of Life Sciences and Systems Biology, University of Turin (Italy). The Accession numbers of the sequences deposited in GenBank are available in the supplementary material 1.

Table 1. Gene loci sequenced, primers for molecular analysis and PCR programs.

Fungi	Gene loci and DNA regions sequenced[a]	Primers (Forward and Reverse)	PCR amplification Conditions	References for primers[b]
Alternaria	*GAPDH*	GPD1 and GPD2	96 °C: 2 min, (96 °C: 1 min, 50 °C: 1 min, 72 °C: 50 sec) × 35 cycles; 72 °C: 5 min	(1)
Aspergillus	*CAL*	CL1 and CL2a	95 °C: 10 min, (95 °C: 50 sec, 55 °C: 50 sec, 72 °C: 1 min) × 35 cycles; 72 °C: 7 min	(2)
Aspergillus, Penicillium, Thelebolus	*TUB*	BT–2a and BT–2b	94 °C: 4 min, (94 °C: 35 sec, 58 °C: 35 sec, 72 °C: 50 sec) × 35 cycles; 72 °C: 5 min	(3)
Cladosporium	*ACT*	ACT–512F and ACT–783R	94 °C: 8 min, (94 °C: 15 sec, 61 °C: 20 sec, 72 °C: 40 sec) × 35 cycles; 72 °C: 10 min	(4)
Yeast like fungi (*Holtermanniella, Metschnikowia, Pseudozyma*, Sporidiobolales)	D1-D2	NL1-NL2	94 °C: 4 min, (94 °C: 1 min, 52 °C: 35 sec, 72 °C: 1.5 min) × 35 cycles; 72 °C: 5 min	(5)
Alternaria, Thelebolus, sterile mycelia and taxa for whom no specific primers are required	ITS	ITS1 and ITS4	95 °C: 5 min, (95 °C:40 s, 55 °C: 50 s, 72 °C: 50 sec) × 35 cycles; 72 °C: 8 min	(6)
Sterile mycelia	LSU	LROR and LR7	95 °C: 5 min, (95 °C: 1 min, 50 °C: 1 min, 72 °C: 2 min) × 35 cycles; 72 °C: 10 min	(7)

[a] *GAPDH*: partial glyceraldehyde-3-phosphate dehydrogenase gene; *CAL*: partial calmodulin gene; *TUB*: partial beta-tubulin gene; *ACT*: partial actin gene; D1-D2: D1-D2 region of the nuclear ribosomal DNA large subunit; ITS: internal transcribed spacer regions and intervening 5.8S nrRNA gene; LSU: partial nuclear ribosomal DNA large subunit.

[b](1) Berbee *et al*. 1999, (2) O'Donnell *et al*. 2000, (3) Glass & Donaldson 1995, (4) Carbone & Kohn 1999, (5) De Barros Lopes *et al*. 1998, (6) White *et al*. 1990, (7) Vilgalys & Hester 1990.

Table 2. Fungal taxa isolated from *D. fragilis* (DF), *P. johnstonia* (PJ) and *S. ciliatum* (SY) and their relative abundance (RA) in percentage. The species already found in marine environment (MA) and associated with sponges (SP) are reported, as well as the first record (FR).

	RA %				
	DF	PJ	SY	MA	SP
Ascomycota					
Acremonium breve	0.6			(20)	FR
Acremonium implicatum	0.6			(1), (2), (3), (4), (5), (19)	(3)
Acremonium persicinum	1.3			(7)	(33)
Acremonium potronii[a]	5.8		1.0	(7), (34)	FR
Acremonium tubakii	5.8			(1), (34)	FR
Acremonium zonatum			2.9	FR	FR
Alternaria molesta[a]		1.4		(30)	FR
Alternaria sp.[a]			1.0	-	-
Aspergillus creber	4.5	2.7		(23)	FR
Aspergillus flavipes		5.4		(22)	(35)
Aspergillus fumigatus	2.6		1.0	(1), (4), (5), (7), (19)	(6), (18), (27)
Aspergillus jensenii	0.6	2.7		FR	FR
Aspergillus puulaauensis	3.8			FR	FR
Aureobasidium pullulans	1.3	2.7		(2), (7), (19), (22), (34)	(9), (36)
Beauveria bassiana	1.9		2.9	(7), (34)	(17), (37)
Bimuria novae-zelandiae[a]	0.6			(31)	FR
Boeremia exigua[a]	0.6			(32), (34)	FR
Botrytis cinerea	0.6			(5), (24), (34)	FR
Cadophora luteo-olivacea	0.6		4.9	(11)	FR
Cladosporium aggregatocicatricatum	0.6			FR	FR
Cladosporium allicinum	2.6	1.4	3.9	(2)	FR
Cladosporium cladosporioides	2.6	6.8	1.0	(1), (2), (5), (7), (19), (21), (22), (34)	(9), (12), (14), (15), (18), (27)
Cladosporium halotolerans		5.4	10.8	(5), (11), (22)	FR

Table 2. (Continued).

	RA %				
	DF	PJ	SY	MA	SP
Cladosporium perangustum	0.6			(25)	FR
Cladosporium pseudocladosporioides	2.6	4.1		(5)	FR
Cladosporium psychrotolerans	1.3			(22)	FR
Cladosporium subtilissimum	0.6			(22)	FR
Cladosporium subuliforme		2.7		FR	FR
Cladosporium xylophilum	0.6			FR	FR
Coniothyrium obiones[a]			1.0	(7)	FR
Cyphellophora sp.[a]			1.0	-	-
Emericellopsis alkalina (asexual morph)	1.3			FR	FR
Emericellopsis maritima (asexual morph)		1.4		(7)	FR
Emericellopsis pallida (asexual morph)	1.3			(7)	FR
Epicoccum nigrum			4.9	(5), (34)	(40)
Fusarium pseudograminearum	0.6			FR	FR
Fusarium solani			1.0	(26)	(3), (41)
Gremmenia infestans[a]			1.0	FR	FR
Hypocreaceae sp.[a]	0.6			-	-
Metschnikowia bicuspidata			5.9	(7), (22)	(38)
Microascaceae sp.[a]	0.6			-	-
Mollisia sp.[a]			1.0	-	-
Myrothecium cinctum[a]	1.3			(39)	(39)
Neocamarosporium betae			1.0	FR	FR
Neocamarosporium calvescens	0.6			FR	FR
Paraphaeosphaeria neglecta (asexual morph)	0.6		1.0	FR	FR
Penicillium antarcticum	10.9	37.8	18.6	(2), (5)	(16)
Penicillium brevicompactum	3.2			(1), (2), (3), (4), (5), (7), (19), (22), (34)	(3), (10), (27)
Penicillium canescens		1.4		(5), (21), (34)	(40)
Penicillium chrysogenum	12.2	13.5		(5), (7), (22), (34)	(3), (6), (10), (27), (40)
Penicillium citreonigrum			1.0	(5), (7), (21)	(40)
Penicillium inflatum	0.6			FR	FR
Penicillium janczewskii	5.8			(7), (34)	FR
Penicillium roqueforti			1.0	(40)	(40)
Penicillium spinulosum		2.7		(1), (7), (34)	FR
Penicillium thomii		1.4		(7), (34)	FR
Penicillium waksmanii	1.3			(7), (34)	(12)
Periconia minutissima			1.0	(7)	FR
Periconia sp.[a]		1.4		-	-
Phaeosphaeria olivacea[a]			1.0	(7)	FR
Phaeosphaeria oryzae[a]			1.0	FR	FR
Phaeosphaeriopsis sp.[a]	0.6		4.9	-	-
Pleosporales sp.[a]			1.0	-	-
Pleosporaceae sp.[a]	0.6			-	-
Pochonia suchlasporia	0.6			(34)	FR
Preussia sp.[a]	0.6			-	-
Pseudeurotium bakeri			1.0	FR	FR
Pseudocercosporella sp.[a]		1.4		-	-
Pyrenochaetopsis microspora[a]	1.9			FR	FR

Table 2. (Continued).

	RA %				
	DF	PJ	SY	MA	SP
Roussoellaceae sp.[a]	0.6			-	-
Sarocladium strictum			14.7	(2), (7), (34)	FR
Scopulariopsis brevicaulis			1.0	(5)	(6), (13)
Thelebolus balaustiformis	0.6			FR	FR
Thelebolus spongiae	0.6			FR	FR
Thyronectria sp.[a]			1.0	-	-
Tilachlidium brachiatum	0.6			(28)	FR
Tolypocladium album	1.9			FR	FR
Tolypocladium cylindrosporum	2.6	1.4	4.9	(29), (34)	FR
Volutella ciliata	0.6			(4)	(40)
Xanthothecium peruvianum	0.6			FR	FR
Basidiomycota					
Bjerkandera sp.[a]	0.6			-	-
Holtermanniella sp.[a]			1.0	-	-
Agarycomycetes sp.[a]	0.6			-	-
Pseudozyma sp.[a]		1.4		-	-
Sporidiobolales sp.[a]		1.4		-	-
Trametes gibbosa[a]	1.3			FR	FR
Mucoromycota					
Absidia glauca	0.6			(19)	FR
Total taxa	54	21	32		
Total exclusive taxa	39	11	21		

(1) Panno *et al*. 2013, (2) Gnavi *et al.* 2017, (3) Paz *et al.* 2010, (4) Costello *et al.* 2001, (5) Bovio *et al.* 2017, (6), Ding *et al.* 2011, (7) Jones *et al.* 2015, (8) Thirunavukkarasu *et al.* 2012, (9) Henríquez *et al.* 2014, (10) Passarini *et al.* 2013, (11) Garzoli *et al.* 2015, (12) Rozas *et al.* 2011, (13) Yu *et al.* 2008, (14) Manriquez *et al.* 2009, (15) San-Martin *et al.* 2005, (16) Park *et al.* 2014, (17) Yamazaki *et al.* 2012, (18) Sayed *et al.* 2016, (19) Oren & Gunde-Cimerman 2012, (20) Kis-Papo *et al.* 2001, (21) Raghukumar & Ravindran 2012, (22) Zajc *et al.* 2012, (23) Jurjevic *et al.* 2012, (24) Suryanarayanan 2012, (25) Liu *et al.* 2016, (26) Hatai 2012, (27) Pivkin *et al.* 2006, (28) Gomes *et al.* 2008, (29) Rämä *et al.* 2014, (30) Tóth *et al.* 2011, (31) Suetrong *et al.* 2009, (32) Di Piazza *et al.* 2017, (33) Fraser *et al.* 2013, (34) Rämä *et al.* 2017, (35) Ratnaweera *et al.* 2016, (36) Shigemori *et al.* 1998, (37) Zhang *et al.* 2017, (38) Baker *et al.* 2009, (39) Wang & Zhu 2008, (40) Wiese *et al.* 2011, (41) Bolaños *et al.* 2015.

[a]Sterile mycelia.

Thelebolus spp. growth conditions and molecular study

Thelebolus spp. MUT 2357 and MUT 2359 were pre-grown on Potato Dextrose Agar - PDA (Potato extract 4 g, dextrose 20 g, agar 15 g, up to 1 L dH_2O) at 25 °C and then inoculated in triplicate onto Petri dishes (9 cm Ø) containing MEA, PDA and Carrot Agar (grated carrot 20 g boiled and filtered, agar 20 g, up to 1 L dH_2O) alone and with different concentration of NaCl (2.5 %, 5 %, 10 %, 15 %) and incubated at 4 °C, 15 °C and 25 °C. The fungal growth, as well as macroscopic and microscopic features, were evaluated at three, seven, 10, 14, 17, 21 d after the inoculum. Mature reproductive structures were observed and photographed with an optical microscope (LEICA DM4500 B) equipped with a camera (LEICA DFC320). Morphological data (micro- and macroscopic) were compared with the available description of *Thelebolus* species. DNA was extracted as mentioned above and the ITS and beta-tubulin regions were amplified as recommended by previous studies (de Hoog *et al.* 2005, Crous *et al.* 2015). A two-marker dataset (supplementary material 2) was built for a phylogenetic analysis, which was performed as described in the previous section.

Statistical analyses

Statistical analyses on the fungal community associated with sponges were performed using PRIMER v. 7.0 (Plymouth Routines In Multivariate Ecological Research; Clarke and Warwick 2001). The Similarity Percentages (SIMPER) analysis mostly highlighted the dissimilarity within the fungal community of the three sponges. The Permutational Multivariate Analysis of Variance (PERMANOVA; pseudo-F index; p<0.05) allowed the differences between the sponge mycobiotas to be assessed. Principal Coordinate Analysis (PCO) visualised data.

RESULTS

The use of different isolation techniques or culture conditions resulted in an increase in the number of fungal isolates. As reported in Fig. 1A the majority of the taxa were isolated exclusively by homogenisation of sponge tissues, while the remaining by directly plating the sponge tissue; less than 18 % were recovered with both techniques. Overall, from 67 % (*P. johnstonia*) to 75 % (*S. ciliatum*)

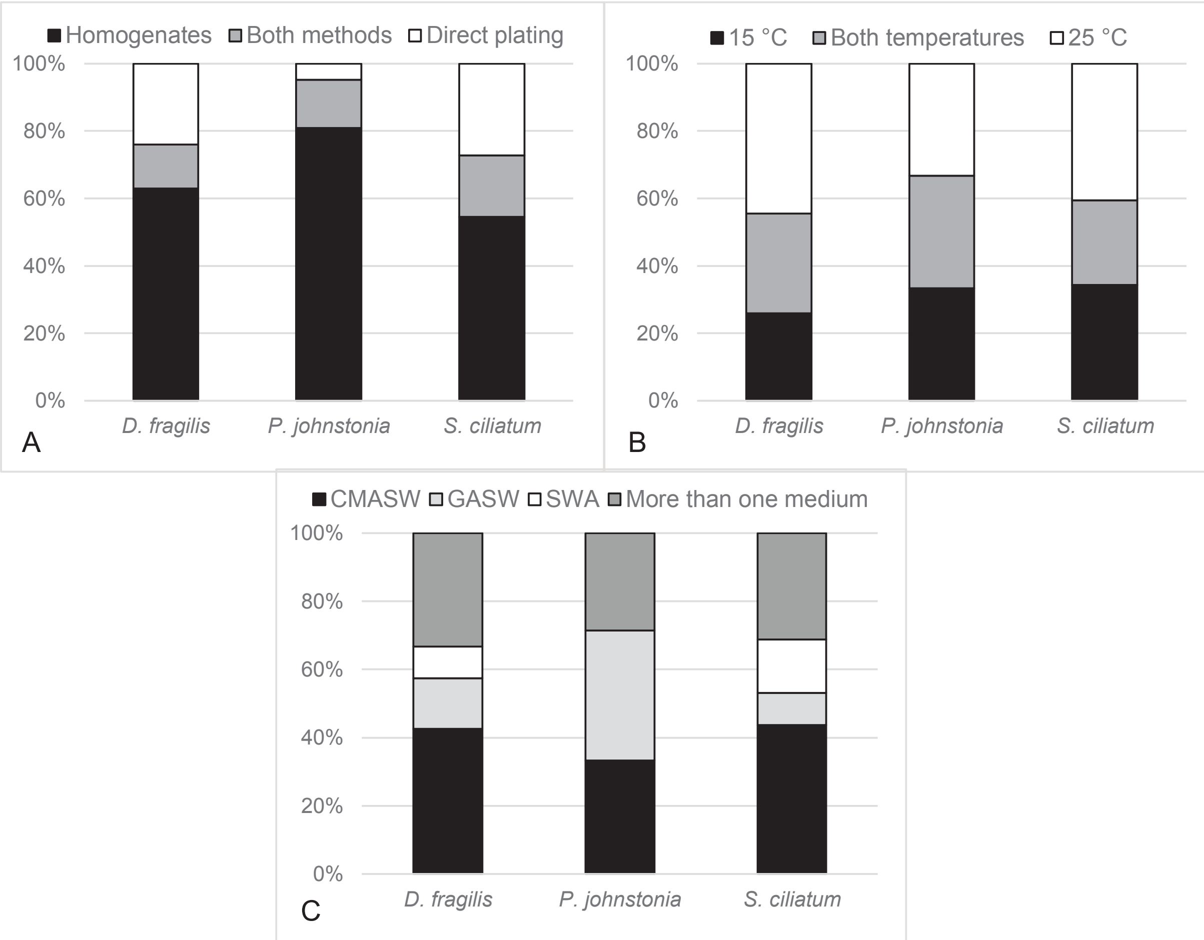


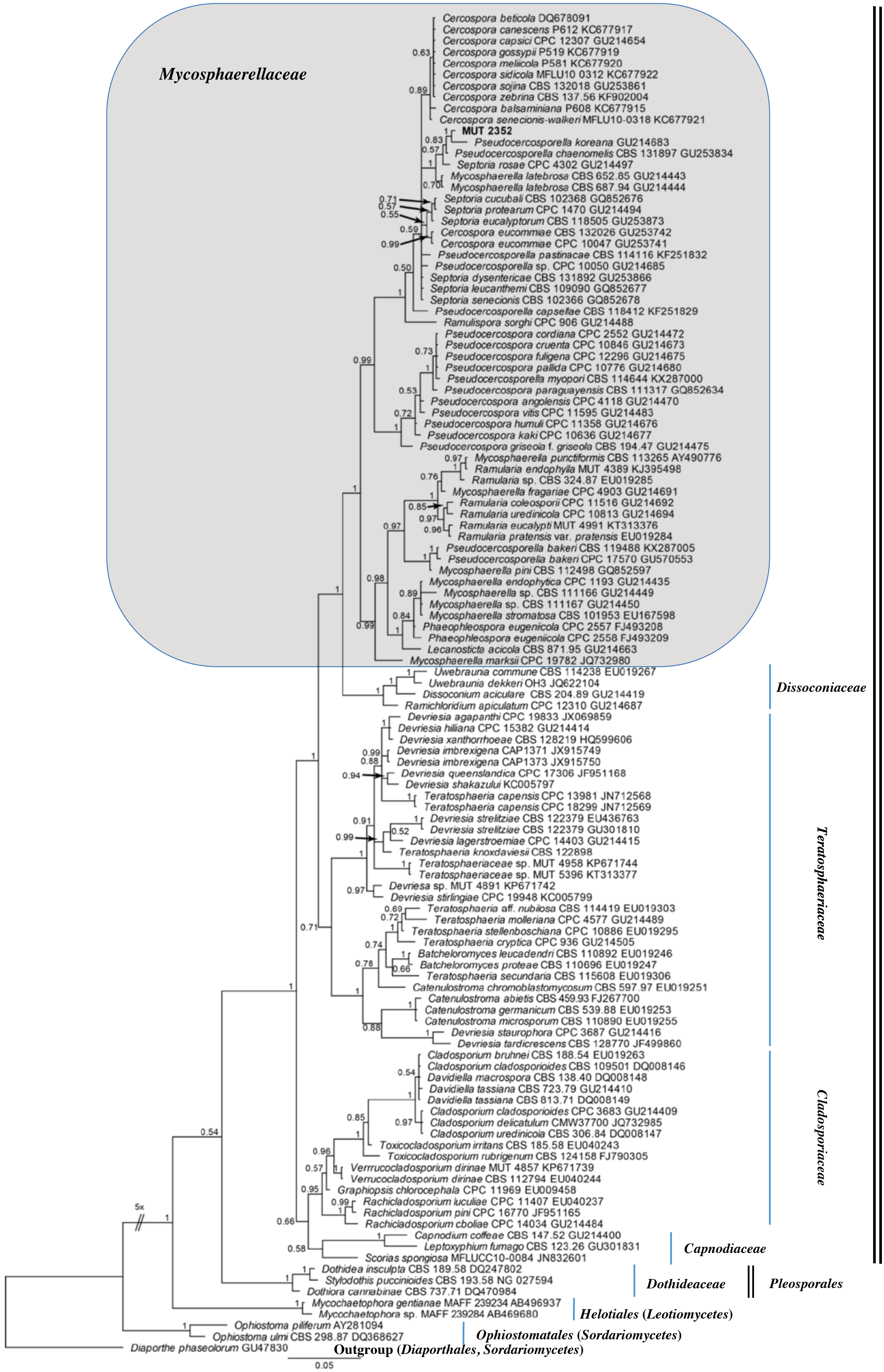
Fig. 1. Influence of **A.** Isolation methods. **B.** Incubation temperatures. **C.** Growth media, on the fungal community associated with *D. fragilis*, *P. johnstonia* and *S. ciliatum*.

of taxa were isolated only on one temperature condition (15 °C or 25 °C) as reported in Fig. 1B. Regarding the growth media (Fig. 1C), almost half of the taxa of *D. fragilis* (43 %) and of *S. ciliatum* (44 %) grew exclusively on CMASW; while 24 % and 25 % of taxa associated with *D. fragilis* and *S. ciliatum* were isolated only on oligotrophic media, mimicking sponges' composition (GASW) or marine water (SWA). The majority of the fungi associated with *P. johnstonia* were only isolated on GASW (38 %) or on CMASW (33 %); no exclusive taxa were reported on SWA. Interestingly, 66 %, 56 % and 38 % of taxa from *S. ciliatum, D. fragilis* and *P. johnstonia*, respectively, not only were recovered in one condition but were isolated only from one plate.

A total of 87 taxa were isolated: 54 taxa from *D. fragilis*, 32 from *S. ciliatum* and 21 from *P. johnstonia*; 79 % of the taxa were recognised at species level, 13 % at genus level, 5 % at family level, 2 % at order level, and 1 % at class level (Table 2). About one third of taxa were sterile despite the attempt to stimulate the production of reproductive structures using different culture media and incubation under near-UV light. Several sterile mycelia (Table 2) showed the same similarity percentages with different species and/or low homology with sequences deposited in public databases. In addition, some cryptic strains belonging to the *Pleosporales* order (MUT 2482, MUT 2870, MUT 2952, MUT 3080 and MUT 2425) presented only the asexual form in axenic culture (for many genera, only the description of the sexual morph is available). For these reasons, a phylogenetic analysis based on LSU region was necessary to achieve their best identification. In detail, *Capnodiales* (Fig. 2) and *Chaetothyriales* (Fig. 3) were represented by one isolate each; 17 strains belonged to *Pleosporales* order (Fig. 4); two and eight strains were grouped in *Leotiomycetes* (Fig. 5) and *Sordariomycetes* (Fig. 6), respectively.

The majority of taxa belonged to *Ascomycota* (92 %), with few representatives of *Basidiomycota* (7 %) and *Mucoromycota* (1 %). The genera *Cladosporium* and *Penicillium* (11 species), *Acremonium* (six species) and *Aspergillus* (five species) were the most represented in terms of species. In terms of first reports, 49 and 21 species were first recorded here as being associated with sponges and the marine environment, respectively (Table 2). Four species (*Cladosporium allicinum*, *Cladosporium cladosporioides*, *Penicillium antarcticum* and *Tolypocladium cylindrosporum)* were common among the three sponges (Fig. 7). *Dysidea fragilis* and *S. ciliatum* shared an additional six species (*Acremonium potronii, Aspergillus fumigatus, Beauveria bassiana, Cadophora luteo-olivacea, Paraphaeosphaeria neglecta, Phaeosphaeriopsis* sp.); while, *D. fragilis* and *P. johnstonia* had five additional species in common (*Aspergillus creber, Aspergillus jensenii, Aureobasidium pullulans, Cladosporium pseudocladosporioides*

Fig. 2. Bayesian phylogram of *Capnodiales* (*Dothideomycetes*) based on rDNA large subunit (LSU). One fungal isolate (MUT 2352) is included and identified as *Cercospora* sp. within the *Mycosphaerellaceae*. Branch numbers indicate BPP values.


Mycosphaerellaceae
Cercospora beticola DQ678091
Cercospora canescens P612 KC677917
Cercospora capsici CPC 12307 GU214654
Cercospora gossypii P519 KC677919
Cercospora meliicola P581 KC677920
Cercospora sidicola MFLU10 0312 KC677922
Cercospora sojina CBS 132018 GU253861
Cercospora zebrina CBS 137.56 KF902004
Cercospora balsaminiana P608 KC677915
Cercospora senecionis-walkeri MFLU10-0318 KC677921
MUT 2352
Pseudocercosporella koreana GU214683
Pseudocercosporella chaenomelis CBS 131897 GU253834
Septoria rosae CPC 4302 GU214497
Mycosphaerella latebrosa CBS 652.85 GU214443
Mycosphaerella latebrosa CBS 687.94 GU214444
Septoria cucubali CBS 102368 GQ852676
Septoria protearum CPC 1470 GU214494
Septoria eucalyptorum CBS 118505 GU253873
Cercospora eucommiae CBS 132026 GU253742
Cercospora eucommiae CPC 10047 GU253741
Pseudocercosporella pastinacae CBS 114116 KF251832
Pseudocercosporella sp. CPC 10050 GU214685
Septoria dysentericae CBS 131892 GU253866
Septoria leucanthemi CBS 109090 GQ852677
Septoria senecionis CBS 102366 GQ852678
Pseudocercosporella capsellae CBS 118412 KF251829
Ramulispora sorghi CPC 906 GU214488
Pseudocercospora cordiana CPC 2552 GU214472
Pseudocercospora cruenta CPC 10846 GU214673
Pseudocercospora fuligena CPC 12296 GU214675
Pseudocercospora pallida CPC 10776 GU214680
Pseudocercosporella myopori CBS 114644 KX287000
Pseudocercospora paraguayensis CBS 111317 GQ852634
Pseudocercospora angolensis CPC 4118 GU214470
Pseudocercospora vitis CPC 11595 GU214483
Pseudocercospora humuli CPC 11358 GU214676
Pseudocercospora kaki CPC 10636 GU214677
Pseudocercospora griseola f. griseola CBS 194.47 GU214475
Mycosphaerella punctiformis CBS 113265 AY490776
Ramularia endophylla MUT 4389 KJ395498
Ramularia sp. CBS 324.87 EU019285
Mycosphaerella fragariae CPC 4903 GU214691
Ramularia coleosporii CPC 11516 GU214692
Ramularia uredinicola CPC 10813 GU214694
Ramularia eucalypti MUT 4991 KT313376
Ramularia pratensis var. pratensis EU019284
Pseudocercosporella bakeri CBS 119488 KX287005
Pseudocercosporella bakeri CPC 17570 GU570553
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Mycosphaerella endophytica CPC 1193 GU214435
Mycosphaerella sp. CBS 111166 GU214449
Mycosphaerella sp. CBS 111167 GU214450
Mycosphaerella stromatosa CBS 101953 EU167598
Phaeophleospora eugeniicola CPC 2557 FJ493208
Phaeophleospora eugeniicola CPC 2558 FJ493209
Lecanosticta acicola CBS 871.95 GU214663
Mycosphaerella marksii CPC 19782 JQ732980
Uwebraunia commune CBS 114238 EU019267
Uwebraunia dekkeri OH3 JQ622104
Dissoconium aciculare CBS 204.89 GU214419
Ramichloridium apiculatum CPC 12310 GU214687
Dissoconiaceae
Devriesia agapanthi CPC 19833 JX069859
Devriesia hilliana CPC 15382 GU214414
Devriesia xanthorrhoeae CBS 128219 HQ599606
Devriesia imbrexigena CAP1371 JX915749
Devriesia imbrexigena CAP1373 JX915750
Devriesia queenslandica CPC 17306 JF951168
Devriesia shakazului KC005797
Teratosphaeria capensis CPC 13981 JN712568
Teratosphaeria capensis CPC 18299 JN712569
Devriesia strelitziae CBS 122379 EU436763
Devriesia strelitziae CBS 122379 GU301810
Devriesia lagerstroemiae CPC 14403 GU214415
Teratosphaeria knoxdaviesii CBS 122898
Teratosphaeriaceae sp. MUT 4958 KP671744
Teratosphaeriaceae sp. MUT 5396 KT313377
Devriesa sp. MUT 4891 KP671742
Devriesia stirlingiae CPC 19948 KC005799
Teratosphaeria aff. nubilosa CBS 114419 EU019303
Teratosphaeria molleriana CPC 4577 GU214489
Teratosphaeria stellenboschiana CPC 10886 EU019295
Teratosphaeria cryptica CPC 936 GU214505
Batcheloromyces leucadendri CBS 110892 EU019246
Batcheloromyces proteae CBS 110696 EU019247
Teratosphaeria secundaria CBS 115608 EU019306
Catenulostroma chromoblastomycosum CBS 597.97 EU019251
Catenulostroma abietis CBS 459.93 FJ267700
Catenulostroma germanicum CBS 539.88 EU019253
Catenulostroma microsporum CBS 110890 EU019255
Devriesia staurophora CPC 3687 GU214416
Devriesia tardicrescens CBS 128770 JF499860
Teratosphaeriaceae
Cladosporium bruhnei CBS 188.54 EU019263
Cladosporium cladosporioides CBS 109501 DQ008146
Davidiella macrospora CBS 138.40 DQ008148
Davidiella tassiana CBS 723.79 GU214410
Davidiella tassiana CBS 813.71 DQ008149
Cladosporium cladosporioides CPC 3683 GU214409
Cladosporium delicatulum CMW37700 JQ732985
Cladosporium uredinicola CBS 306.84 DQ008147
Toxicocladosporium irritans CBS 185.58 EU040243
Toxicocladosporium rubrigenum CBS 124158 FJ790305
Verrrucocladosporium dirinae MUT 4857 KP671739
Verrucocladosporium dirinae CBS 112794 EU040244
Graphiopsis chlorocephala CPC 11969 EU009458
Rachicladosporium luculiae CPC 11407 EU040237
Rachicladosporium pini CPC 16770 JF951165
Rachicladosporium cboliae CPC 14034 GU214484
Cladosporiaceae
Capnodium coffeae CBS 147.52 GU214400
Leptoxyphium fumago CBS 123.26 GU301831
Scorias spongiosa MFLUCC10-0084 JN832601
Capnodiaceae
CAPNODIALES
Dothidea insculpta CBS 189.58 DQ247802
Stylodothis puccinioides CBS 193.58 NG 027594
Dothiora cannabinae CBS 737.71 DQ470984
Dothideaceae
Pleosporales
Mycochaetophora gentianae MAFF 239234 AB496937
Mycochaetophora sp. MAFF 239284 AB469680
Helotiales (Leotiomycetes)
Ophiostoma piliferum AY281094
Ophiostoma ulmi CBS 298.87 DQ368627
Ophiostomatales (Sordariomycetes)
Diaporthe phaseolorum GU47830
Outgroup (Diaporthales, Sordariomycetes)
5x
0.05

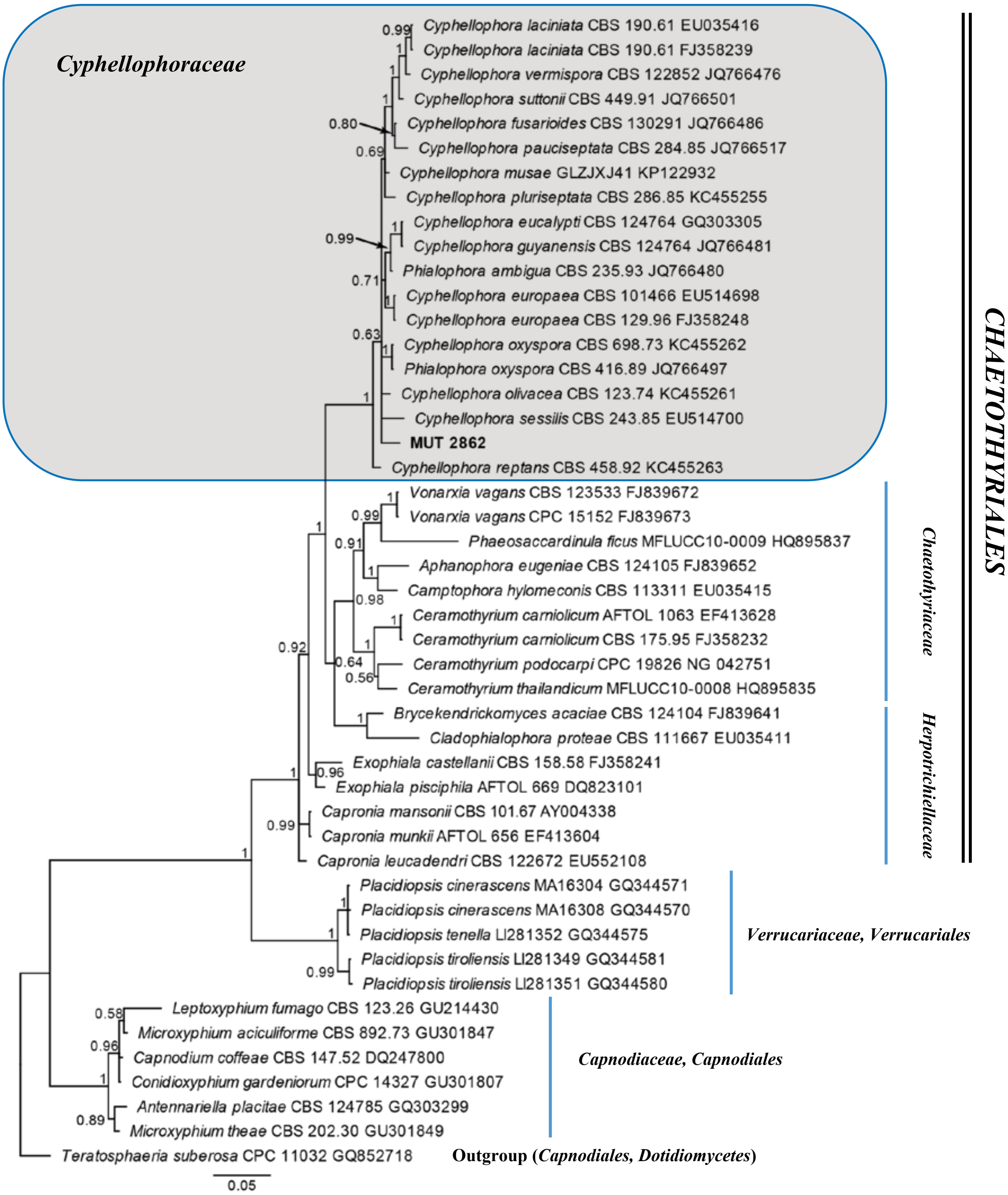

Fig. 3. Bayesian phylogram of *Chaetothyriales* (*Eurotiomycetes*) based on rDNA large subunit (LSU). MUT 2862 is included and clusters within the genus *Cyphellophora*. Branch numbers indicate BPP values.

and Penicillium chrysogenum). *Sycon ciliatum* and *P. johnstonia* shared one additional species (*Cladosporium halotolerans*).

Despite this species overlap, the three sponges host specific fungal communities (Fig. 7); *Dysidea fragilis* mycobiota was represented by 72.2 % exclusive taxa, followed by *S. ciliatum* (65.6 %) and *P. johnstonia* (52.4 %). The specificity of the sponge-mycobiota association was highlighted also by the Permanova analysis that reported a significant difference ($p = 0.011$) among sponges. Almost 45 % of the multivariate variability via two-dimensional Principal Coordinate Analysis (PCO) can be explained by the different fungal communities associated with the sponges (Fig. 8). The dissimilarity among the sponges, highlighted by the SIMPER analysis, was higher between *P. johnstonia* and *S. ciliatum* (89.9 %), with the major contribution given by *P. chrysogenum*, *C. pseudocladosporioides* and *C. luteo-olivacea*. The dissimilarity value of *S. ciliatum* and *D. fragilis* was still high (87.4 %) and *A. potronii*, *Sarocladium strictum* and *Cladosporium psychrotolerans* mostly contribute to the value. The lowest dissimilarity was between *D. fragilis* and *P. johnstonia* (82.6 %), with the major contribution given by *A. potronii*, *C. psychrotolerans* and *Tolypocladium album*.

Overall, *D. fragilis* presented the most diverse mycobiota, including two fungi MUT 2357 and MUT 2359, attributed to the genus *Thelebolus* both by molecular and morphological analyses. No matches in morphological features were observed among our strains and the 16 species and two varieties of *Thelebolus* known (CBS-KNAW Collection, Westerdijk Fungal Biodiversity Institute, MycoBank). The phylogenetic tree (Fig. 9) based on two markers (ITS and beta-tubulin) confirmed the uniqueness of *Thelebolus* MUT 2357 and *Thelebolus* MUT 2359.

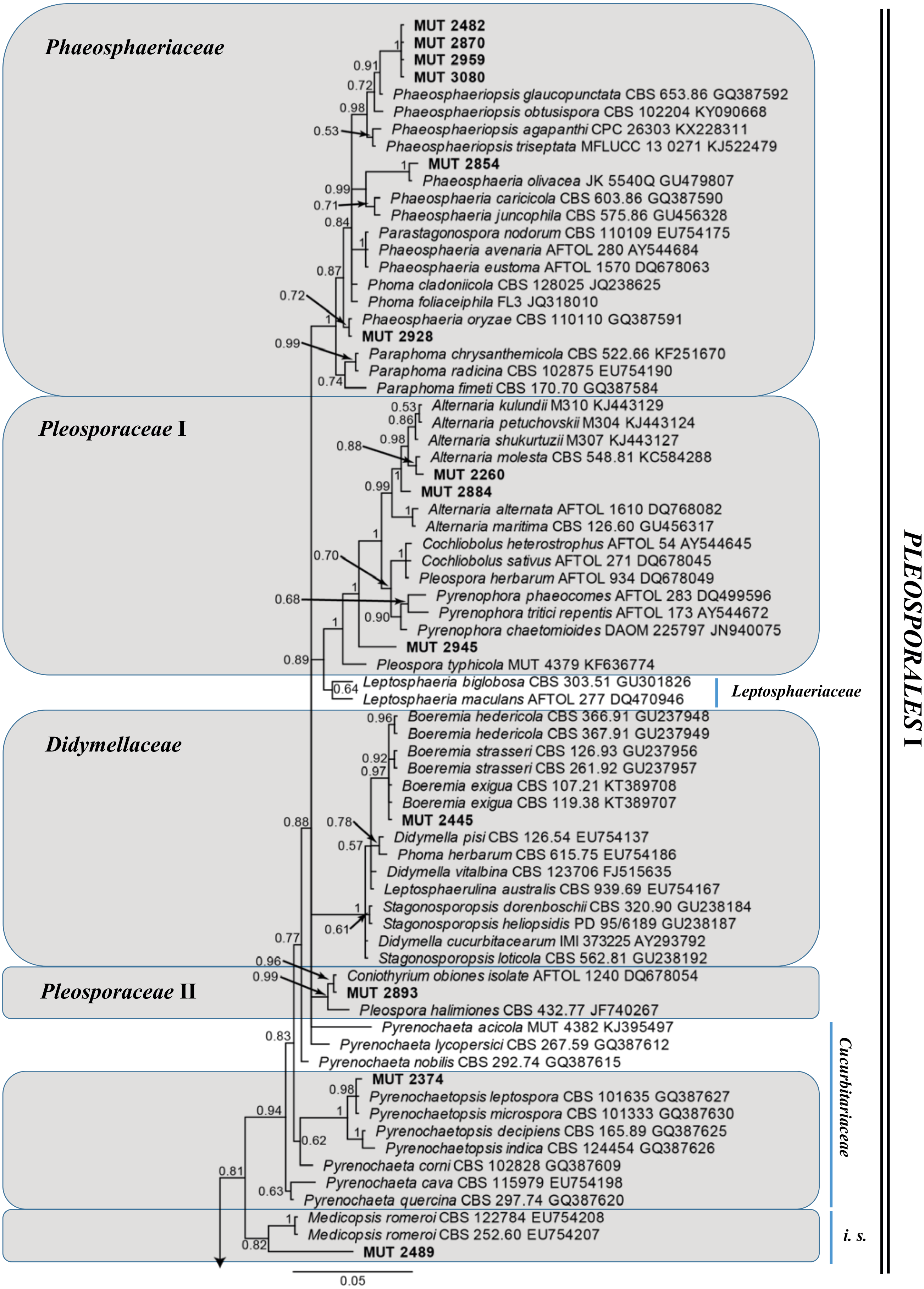

Fig. 4. Bayesian phylogram of *Pleosporales* (*Dothideomycetes*) based on rDNA large subunit (LSU). Six and four fungal isolates clustered within the *Phaeosphaeriaceae* and the *Pleosporaceae*, respectively. Six fungal taxa clustered individually within the *Didymellaceae*, *Cucurbitariaceae*, *Montagnulaceae*, *Periconiaceae*, *Sporormiaceae* and *Roussoellaceae*/*Thyridariaceae*. One fungus was included in the *Pleosporales* order. Branch numbers indicate BPP values. *i.s.* = *incertae sedis*.

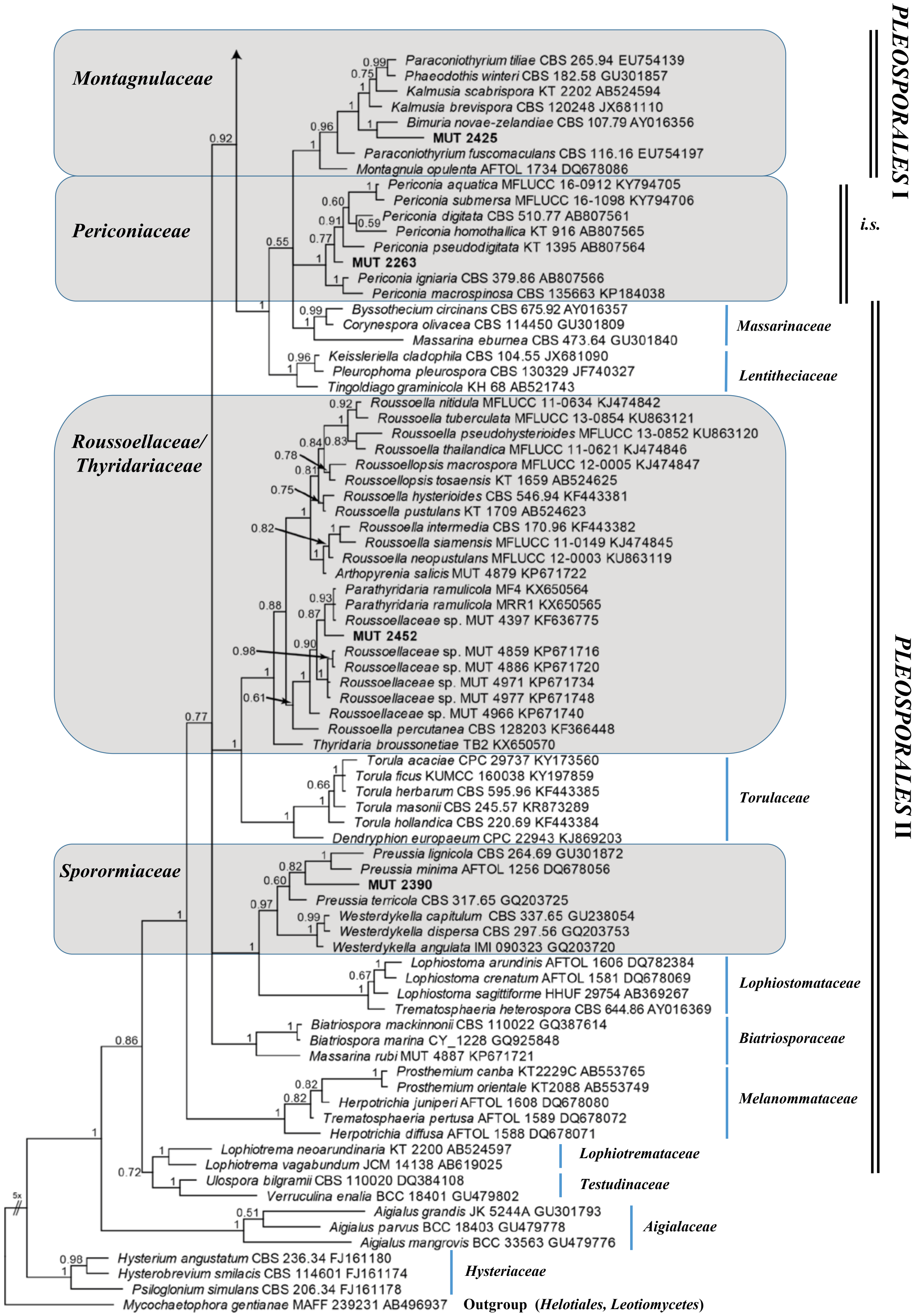

Fig. 4. (Continued).

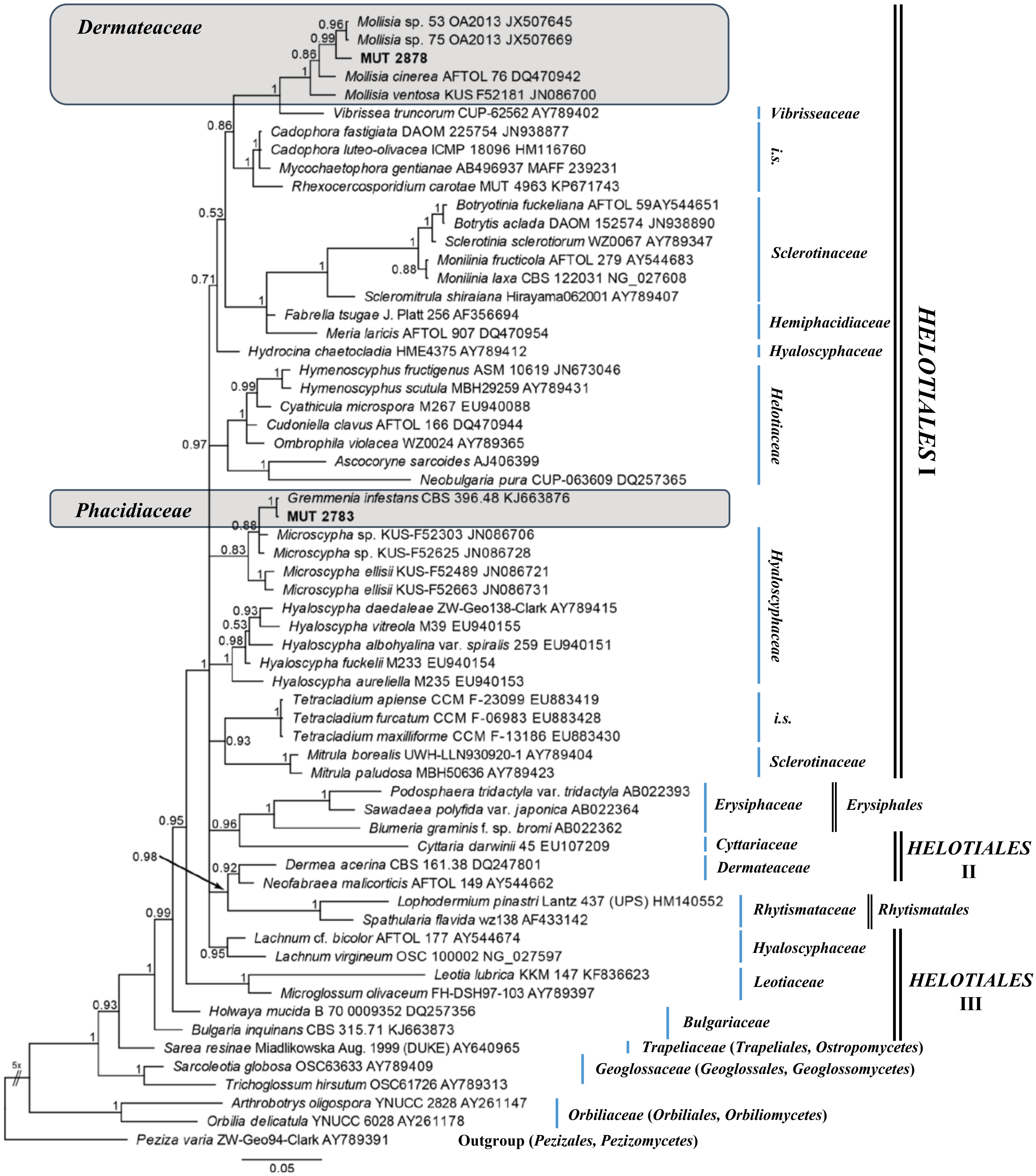

Fig. 5. Bayesian phylogram of *Leotiomycetes* based on rDNA large subunit (LSU). Two fungal isolates were identified as *Mollisia* sp. and *Gremmenia infestans* within the *Dermateaceae* and *Phacidiaceae*, respectively. Branch numbers indicate BPP values.

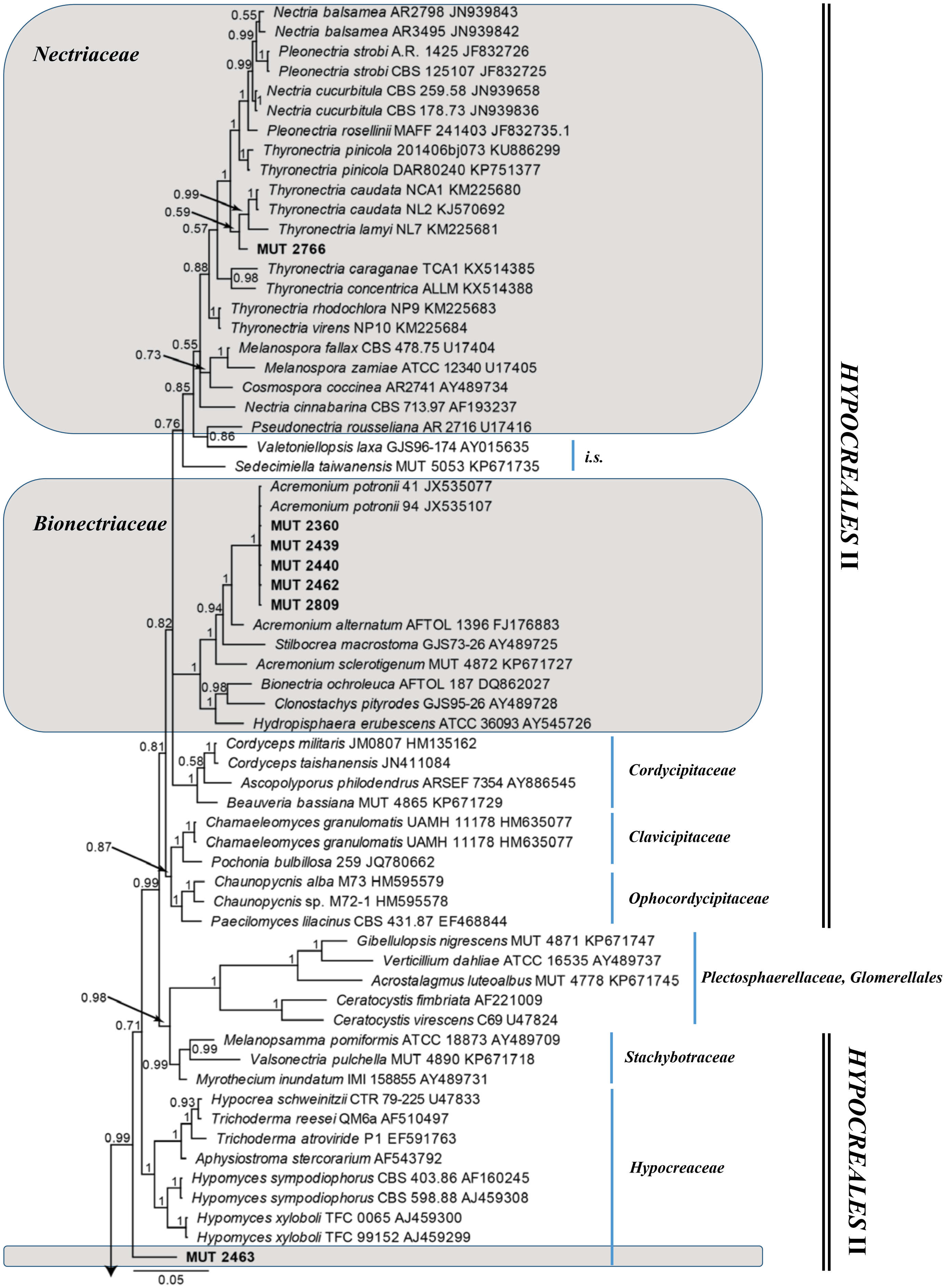

Fig. 6. Bayesian phylogram of *Sordariomycetes* based on rDNA large subunit (LSU). Three fungal taxa clustered individually within the *Nectriaceae*, *Hypocreaceae* and *Microascaceae*. Five fungal isolates clustered within the *Bionectriaceae*. Branch numbers indicate BPP values.

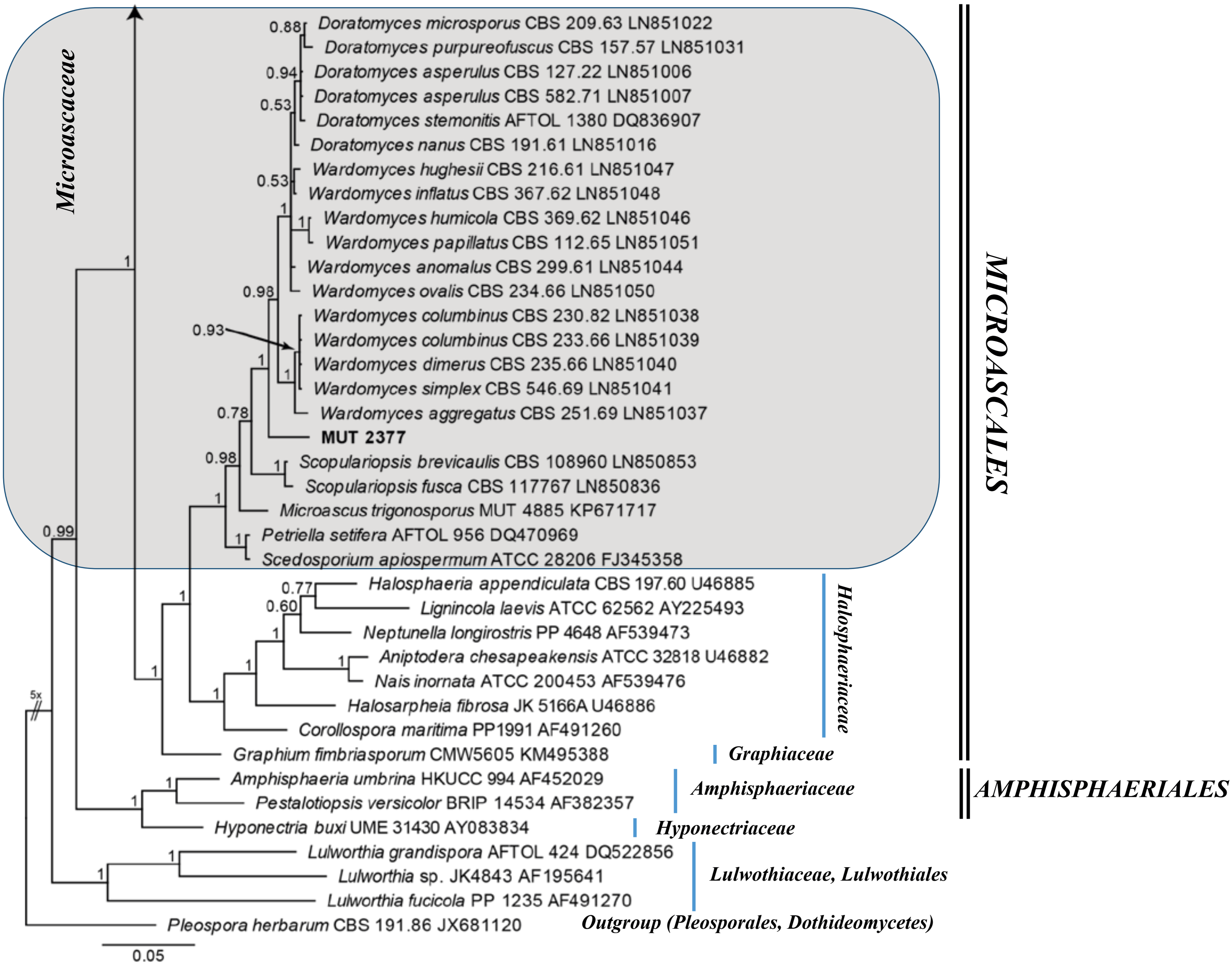

Fig. 6. (Continued).

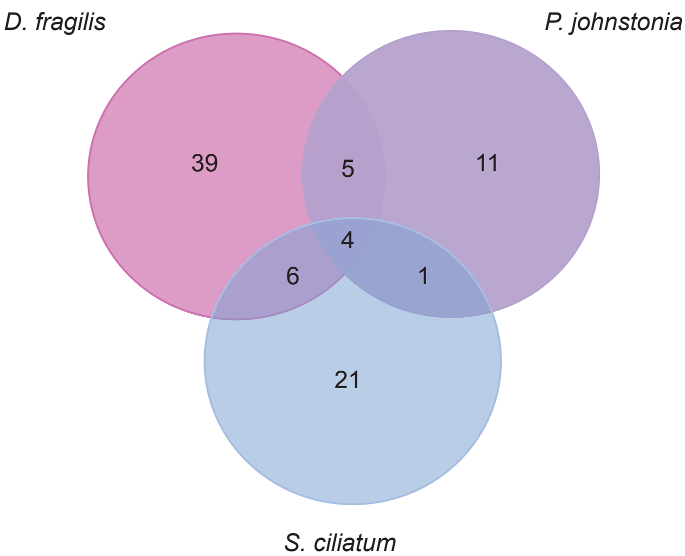

Fig. 7. Exclusive and common fungal taxa species occurring in *D. fragilis, S. ciliatum* and *P. johnstonia*

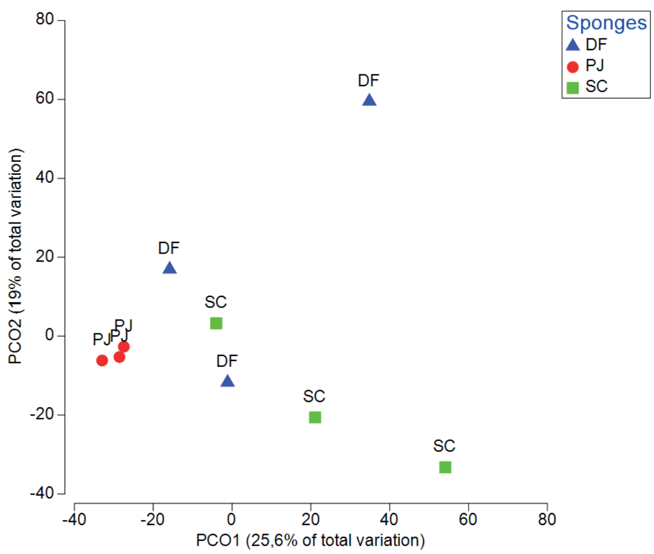

Fig. 8. PCO on the fungal communities of the three Atlantic sponges *D. fragilis* (DF), *P. johnstonia* (PJ) and *S. ciliatum* (SC).

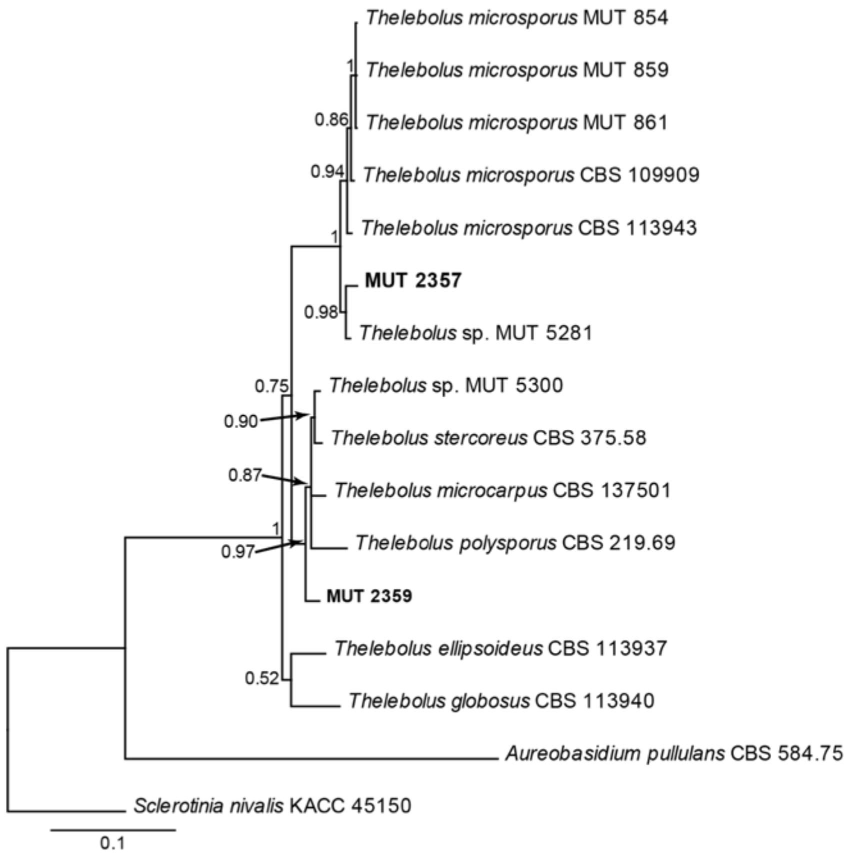

Fig 9. Bayesian phylogram of the genus *Thelebolus* based on a combined dataset of ITS and beta-tubulin partial sequences. MUT 2357 and MUT 2359 were identified as new species, *T. balaustiformis* and *T. spongiae*, respectively. Branch numbers indicate BPP values

Taxonomy

Classification: *Thelebolaceae*, *Thelebolales*, *Leotiomycetes.*

Thelebolus balaustiformis E. Bovio, L. Garzoli, A. Poli, V. Prigione, G.C. Varese, ***sp. nov***. MycoBank MB824102. Figs 10–13.

Etymology: The specific epithet *balaustiformis* is derived from the similarity of ascomata, either whole or in section, with the pomegranate (*Punica granatum*) fruit, which, in botanical terms, is called *balaustium*.

Ascomata were produced only on MEA at 4 °C, after 3 wk of incubation (Fig. 10). *Mycelium* hyaline to pale yellow consisting of irregularly swollen, septate hyphae 1.5–5 µm wide. *Ascomata* hyaline or pale yellow, partially immersed in the colony, (87–)100–120 × 100 µm, at first subglobose cleistohymenial then opening by rupturing of the cortical excipulum in the upper part and becoming semiglobular, appearing 'apothecioid' at maturity. *Hymenium* with a palisade of asci. *Cortical excipulum ca*. 6–10 µm thick, consisting of several layers of flattened cells (*textura epidermoidea*). *Asci* 20–30 per ascoma, broadly clavate, rather thick-walled (1–1.5 µm), 48–64-spored, 11–20 × 43–57 µm. *Ascospores* irregularly disposed, ellipsoid with rounded ends (length/width ratio 1.4–1.6), 4–4.7 × 2.8–3 µm, hyaline with a homogenous content, smooth-walled, without mucilaginous substance. *Spores* are forcefully discharged as a single projectile through the subapical part of the ascus. *Paraphyses* absent. *Asexual morph* not observed.

Colony description and physiological features: Colonies on CA attaining 12–17 mm diam in 21 d at 25 °C, plane, thin, mycelium mainly submerged, margins irregular (also at 5 % NaCl), becoming regular in presence of 2.5 % NaCl; at 15 °C and 4 °C colonies very similar with regular margins, reaching 56–59 mm and 36–37 mm diam in 21 d, respectively. The sizes of the colonies (diam in mm) at different salt concentrations and temperature are shown in Fig. 14A–C; the morphologies in Fig. 11. Colonies on PDA attaining 8–9 mm diam in 21 d at 25 °C, developing in height, pink to orange, reverse of the same colour of the surface. At 15 °C colonies reaching 65–73 mm diam in 21 d, plane, pink-orange, margins regular (slightly irregular at 5 % NaCl), slimy; reverse of the same colour of the surface. At 4 °C colonies very similar, reaching 42–48 mm diam in 21 d. Colonies' sizes (diam in mm) and morphologies are shown in Fig. 14D–F and Fig. 12, respectively. Colonies on MEA not growing at 25 °C in 21 d; in presence of 2.5 % and 5 % NaCl, mycelium developing in height, pale orange, attaining 11–13 mm (2.5 % NaCl) and 8–11 mm (5 % NaCl) diam in 21 d. At 15 °C and 4 °C colonies plane, pink, reverse as the surface, reaching 46–48 mm and 25–27 mm diam in 21 d, respectively; Fig. 14 (G–I) report the growth curves (diam in mm); Fig. 13 show the colonies morphologies.

Thelebolus balaustiformis reached the optimal growth at 15 °C, regardless of media and/or salt concentrations utilised (Fig. 14); 25 °C was the most inhibiting temperature (Fig. 14). Regarding the salt concentration, the fungus grew up to 10 % NaCl only on CA at 4 °C (Fig. 14A) and 15 °C (Fig. 14B). On CA at 4 °C and 15 °C the faster growth was reached at 2.5 % NaCl, followed by 0 %, 5 % and 10 % NaCl (Fig. 14A, B). At 25 °C the media with NaCl (2.5 % and 5 %) better supported the fungal growth (Fig. 14C). On PDA at 4 °C the growth was faster with the decreasing of salt (until 0 % NaCl). At 15 °C the conditions with 0 % and 2.5

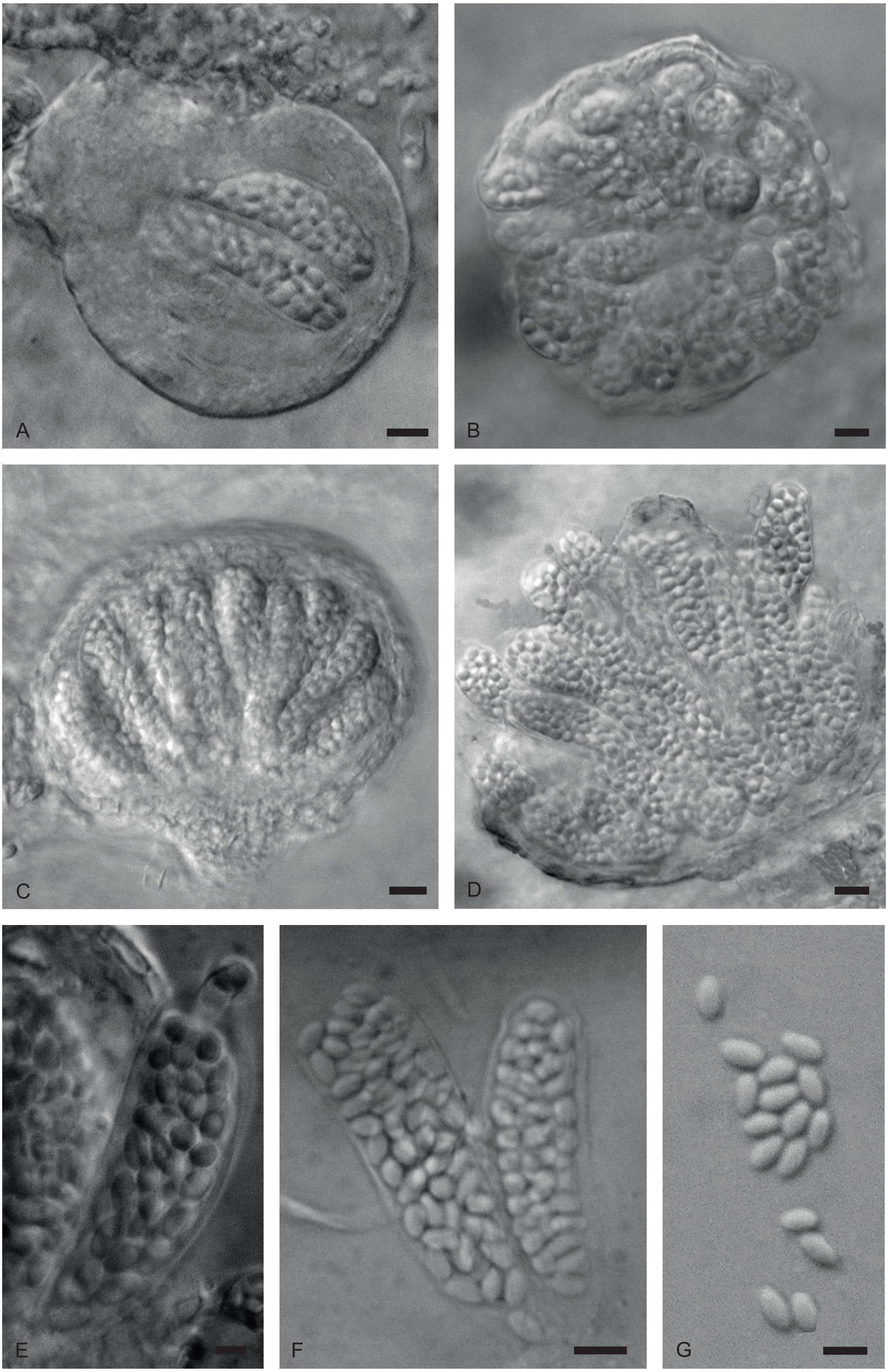

Fig. 10. *Thelebolus balaustiformis* MUT 2357. **A, B.** Closed subglobosus ascoma in the first stage of development. **C.** Ascoma becoming apothecial with mature asci. **D.** Apothecial ascoma with cortical excipulum dehiscent. **E, F.** Mature asci with 48–64 ascospores. **G.** Ascospores. Scale bars: A–D, F = 10 µm; E, G = 5 µm.

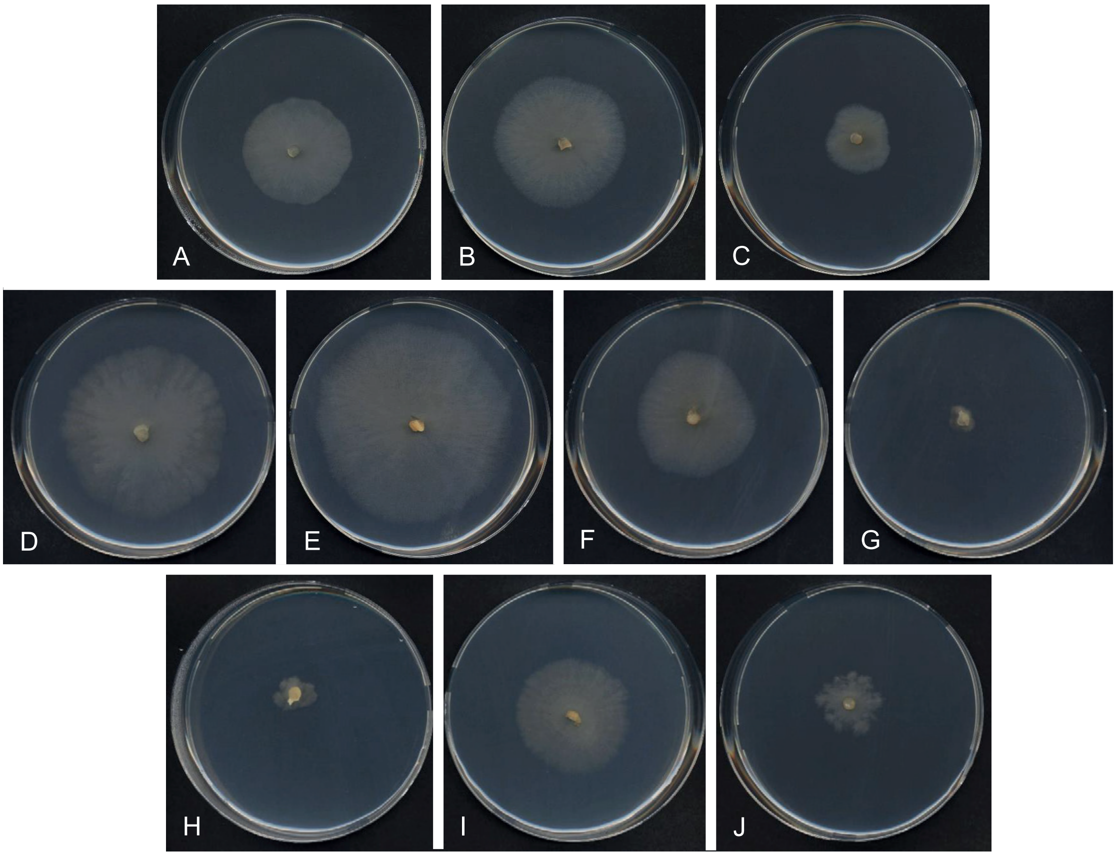

Fig. 11. *Thelebolus balaustiformis* MUT 2357: 21-d-old colonies on CA at 4 °C with **A.** 0 % NaCl, **B.** 2.5 % NaCl, **C.** 5 % NaCl; at 15 °C with **D.** 0 % NaCl, **E.** 2.5 % NaCl, **F.** 5 % NaCl, **G.** 10 % NaCl; at 25 °C with **H.** 0 % NaCl, **I.** 2.5 % NaCl, **J.** 5 % NaCl.

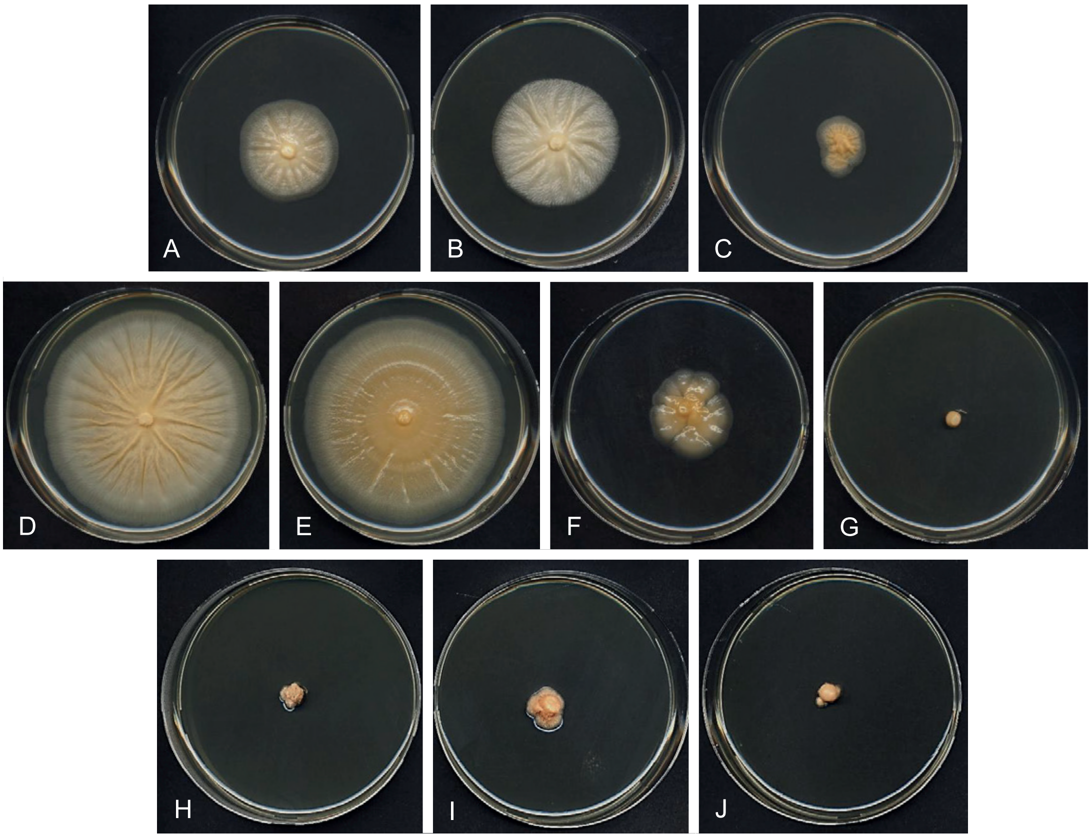

Fig. 12. *Thelebolus balaustiformis* MUT 2357: 21-d-old colonies on PDA at 4 °C with **A.** 0 % NaCl, **B.** 2.5 % NaCl, **C.** 5 % NaCl; at 15 °C with **D.** 0 % NaCl, **E.** 2.5 % NaCl, **F.** 5 % NaCl, **G.** 10 % NaCl; at 25 °C with **H.** 0 % NaCl, **I.** 2.5 % NaCl, **J.** 5 % NaCl.

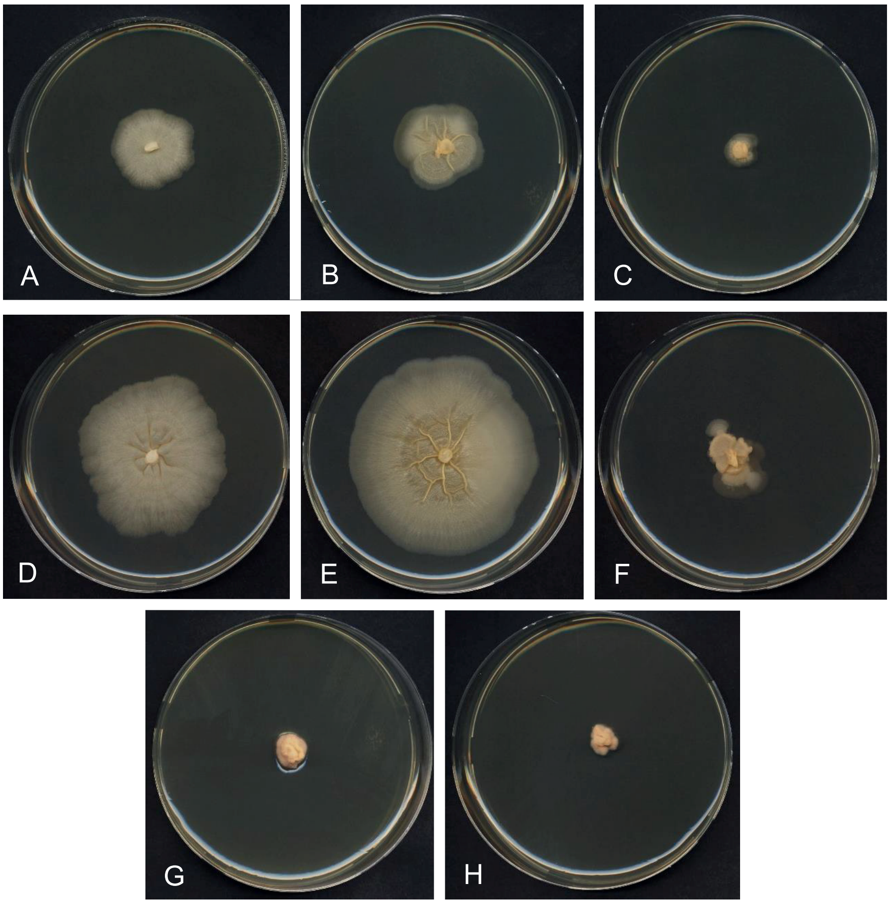

Fig. 13. *Thelebolus balaustiformis* MUT 2357: 21-d-old colonies on MEA at 4 °C with **A.** 0 % NaCl, **B.** 2.5 % NaCl, **C.** 5 % NaCl; at 15 °C with **D.** 0 % NaCl, **E.** 2.5 % NaCl, **F.** 5 % NaCl; at 25 °C with **G.** 2.5 % NaCl, **H.** 5 % NaCl.

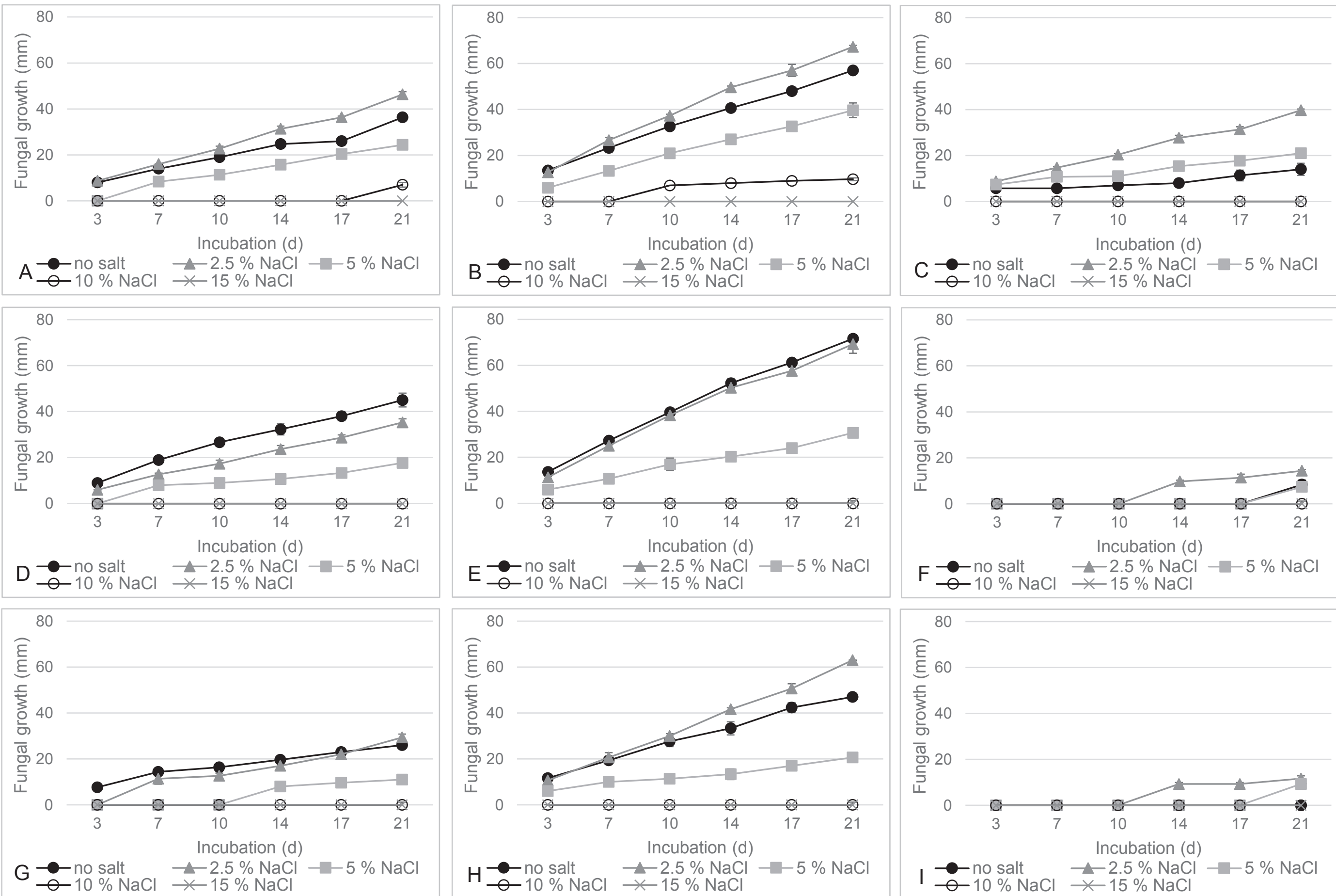

Fig. 14. *Thelebolus balaustiformis* MUT 2357 growth curve with no and different NaCl concentrations on CA at **A.** 4 °C, **B.** 15 °C, **C.** 25 °C; on PDA at **D.** 4 °C, **E.** 15 °C, **F.** 25 °C; on MEA at **G.** 4 °C, **H.** 15 °C, **I.** 25 °C.

% NaCl were comparable, while the slower growth was observed at 5 % NaCl (Fig. 14E). At 25 °C the fungus displayed a similar behaviour compared to CA at the same temperature, the growth was slow and better supported by salt (Fig. 14F). The growth of *T. balaustiformis* on MEA at 4 °C (Fig. 14G), 15 °C (Fig. 14H) and 25 °C (Fig. 14I) was similar to CA (in the same conditions), but there was a less evident difference between 0 % and 2.5 % NaCl at 4 °C, that became more evident at 15 °C, with a faster growth.

Specimen examined: **Ireland**, Galway, Gurraig Sound, Co. Galway, N 53°, 18.944; W 09°, 40.140, on the sponge *Dysidea fragilis*, 4 Jun. 2015, *G. McCormack & D. Firsova.* Holotype preserved as metabolically inactive culture MUT 2357.

Note: *Thelebolus balaustiformis* MUT 2357 was isolated by homogenisation of sponge tissues on CMASW, incubated at 15 °C.

Thelebolus spongiae E. Bovio, L. Garzoli, A. Poli, V. Prigione, G.C. Varese, ***sp. nov***. MycoBank MB824103. Figs 15–18.

Etymology: The specific epithet *spongiae* is derived from the isolation of the fungus from a marine sponge and its strict association with it, due to the isolation by direct plating of the sponge.

Ascomata were produced only on PDA at 4 °C, after 3 wk of incubation (Fig. 15). *Mycelium* hyaline consisting of septate hyphae 3.2–4.7 µm wide, sometimes organised into bundles. *Ascomata* hyaline, superficial, scattered to grouped, from 50 × 40 µm for uni-ascal to 250 × 200 µm diam for multi-ascal, globose to subglobose cleistohymenial not becoming "apothecioid" with age. *Cortical excipulum* clearly differentiated, pale, 6–7 µm thick of 1–2 layers of flattened cells (*textura epidermoidea*). *Asci* 1–6 per ascoma, from globular to sacciform, rather thick-walled (1.5–3 µm), containing hundreds of spores, 37–57 × 50–70 µm. *Ascospores* irregularly disposed, ellipsoid with rounded ends (length/width ratio 2.2–2.4), 7–9.5 × 3.2–4 µm, hyaline with a homogenous content, smooth-walled, without mucilaginous substance. *Paraphyses* absent. *Asexual morph* not observed.

Colony description and physiological features: Colonies on CA attaining 47–51 mm diam in 21 d at 15 °C, smooth, mycelium sparse, pale pink, margins irregular also in presence of NaCl, reverse of the same colour of the surface. At 15 °C colonies similar but with more regular margins, reaching 49–50 mm diam in 21 d. Colonies with regular margins at 4 °C, 28–30 mm diam in 21 d. The sizes of the colonies (diameters in mm) and the morphologies at different salt concentrations and temperature are shown in Fig. 19A–C and Fig. 16, respectively. Colonies on PDA attaining 70–74 mm diam in 21 d at 25 °C, smooth, pale pink, radially sulcate (also in presence of 2.5 % NaCl, not with 5 % NaCl), margins mainly submerged; reverse of the same colour of the surface. At 15 °C and 4 °C colonies very similar, reaching 60–64 mm and 35–38 mm diam in 21 d, respectively. Colonies not radially sulcate, mucoid at 4 °C. Colonies' sizes (diam in mm) and morphologies are shown in Fig. 19D–F and Fig. 17, respectively. Colonies on MEA 30–32 mm diam in 21 d at 25 °C, smooth, mycelium partially submerged, pale pink, margins irregular (regular in presence of 2.5 % and 5 % NaCl); reverse of the same colour of the surface. At 15 °C and 4 °C colonies very similar with margins only slightly irregular, reaching 28–29 mm and 16 mm diam in 21 d, respectively. Fig. 19 (G–I) report the growth curves (diam in mm); Fig. 18 shows the colonies morphologies.

Thelebolus spongiae, as reported in Fig. 19, grew without NaCl and at 2.5 % and 5 % of NaCl; while at 10 % NaCl exhibited no growth, with the exception of PDA at 15 °C where the growth started 17 d after the inoculum and reached 7–9 mm diam in 21 d (Fig. 19E). The fungus grew better with the increasing of the incubation temperature, from 4°C to 25 °C. On PDA and CA at all temperatures, the growth of *T. spongiae* without and with 2.5 % NaCl was comparable (Fig. 19A–F); only at 25 °C on CA the difference was more pronounced: after 10 d the fungus started to grow faster with 2.5 % NaCl (Fig. 19C). The presence of 5 % NaCl made slower *T. spongiae* growth. *T. spongiae* grew faster on MEA in the presence of NaCl (2.5–5 %) compared to its absence; this difference was evident since the firsts stage of development at 15 °C and 25 °C (Fig. 19H, I), while at 4 °C it took 10 d to take shape (Fig. 19G).

Specimen examined: **Ireland**, Galway, Gurraig Sound, Co. Galway, N 53°, 18.944; W 09°, 40.140, on the sponge *Dysidea fragilis*, 4 Jun. 2015, *G. McCormack & D. Firsova.* Holotype preserved as metabolically inactive culture MUT 2359.

Note: *Thelebolus spongiae* MUT 2359 was isolated by direct plating of the sponge on SWA plate and incubated at 15 °C.

DISCUSSION

Isolation techniques

Our study clearly demonstrates the astonishing diversity of fungi inhabiting marine environments: 87 taxa were isolated from three sponges, many of them representing new records in marine ecosystems. This was chiefly due to the use of different isolation techniques and culture conditions. The homogenisation of sponge tissues yielded the highest number of taxa compared to the direct plating. This could be due to the specific requirements of marine fungi and the technique itself. Direct plating resulted in the isolation of only one fungus for each piece of sponge plated; on the contrary, the homogenization best suits the isolation of more marine fungi. These results are in agreement with other comparative studies (Paz *et al.* 2010, Sayed *et al.* 2016). Noteworthy, the direct plating, even if performing less well, allowed the isolation of fungi that otherwise would have not been recorded.

As for the isolation techniques, the use of three different media, also mimicking marine environment and sponge composition resulted in an increase of the number of cultivable fungi. The best performing condition for both *D. fragilis* and *S. ciliatum* was CMASW, a complete medium able to support fungal growth, not extremely rich in nutrients but containing sea salts to provide a condition as much as possible similar to marine environment. Interestingly, the medium that yielded the higher number of isolates in *P. johnstonia* was the gelatine-based medium, specially developed in this research to mimic the host organisms. Usually, media rich in nutrients allow for the isolation of a high number of fungi, but this not necessary means a high biodiversity (Caballero-George *et al.* 2013).

We considered the possible influence of temperature in the isolation of marine fungi from sponges. To mimic marine conditions as much as possible, two different temperatures were set: 25 °C commonly used to culture fungi and 15 °C closer to the environmental conditions of the sponge sampling sites.

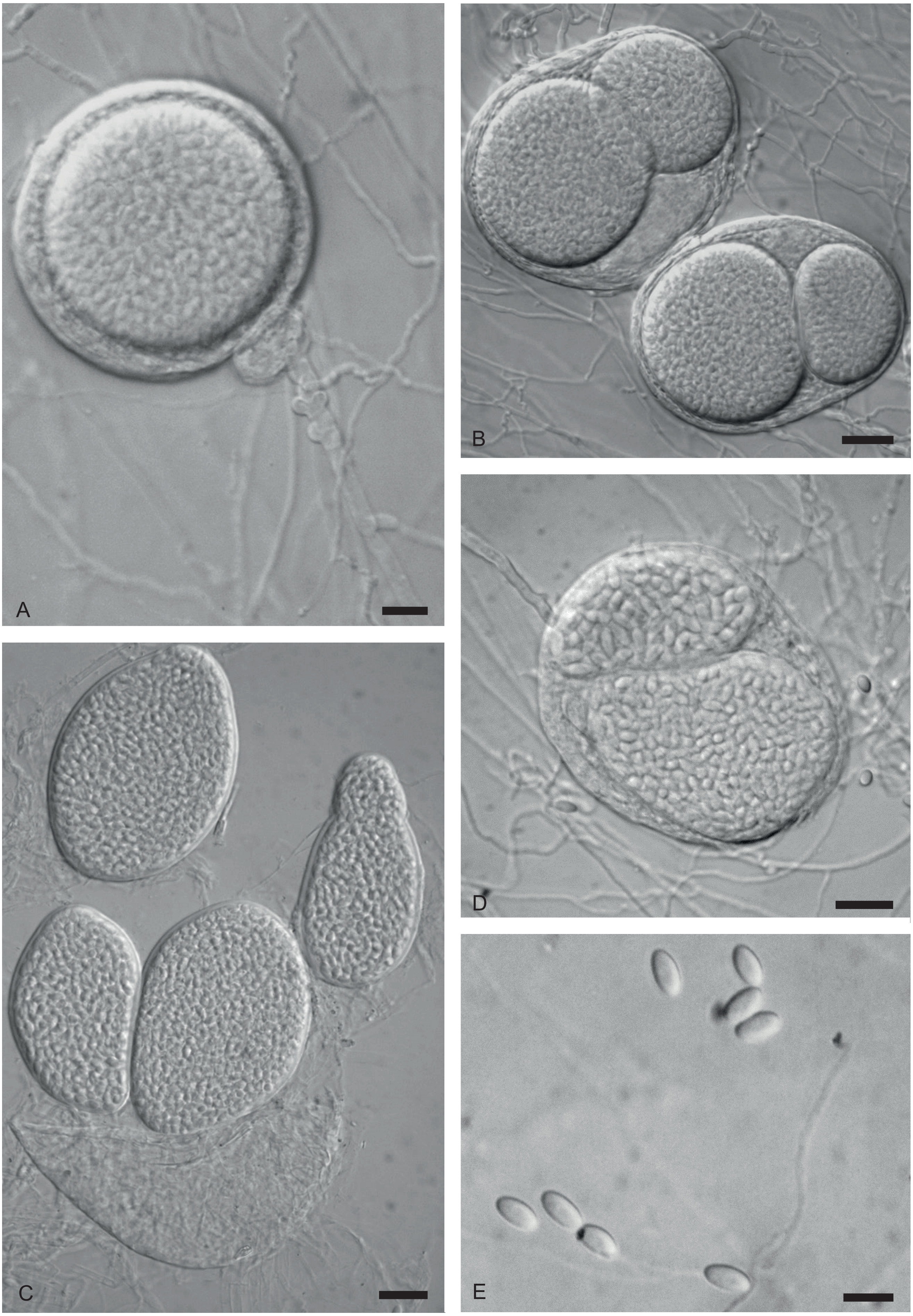

Fig. 15. *Thelebolus spongiae* MUT 2359: **A.** Initial ascoma. **B.** Ascomata with two globular asci. **C.** Mature ascoma opening with four asci. **D.** Ascoma with two sacciform asci. **E.** Ascospores. Scale bars: A, E = 10 µm; B–D = 30 µm.

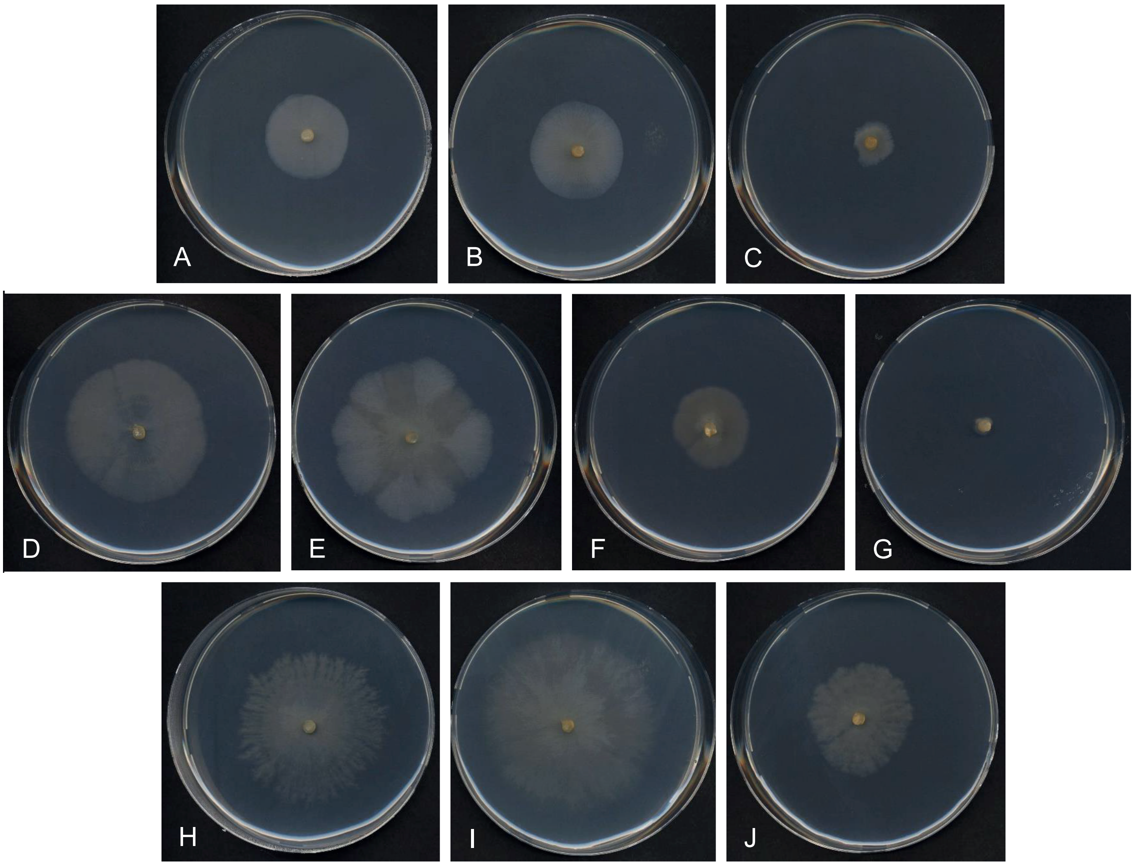

Fig. 16. *Thelebolus spongiae* MUT 2359: 21-d-old colonies on CA at 4 °C with **A.** 0 % NaCl, **B.** 2.5 % NaCl, **C.** 5 % NaCl; at 15 °C with **D.** 0 % NaCl, **E.** 2.5 % NaCl, **F.** 5 % NaCl, **G.** 10 % NaCl; at 25 °C with **H.** 0 % NaCl, **I.** 2.5 % NaCl, **J.** 5 % NaCl.

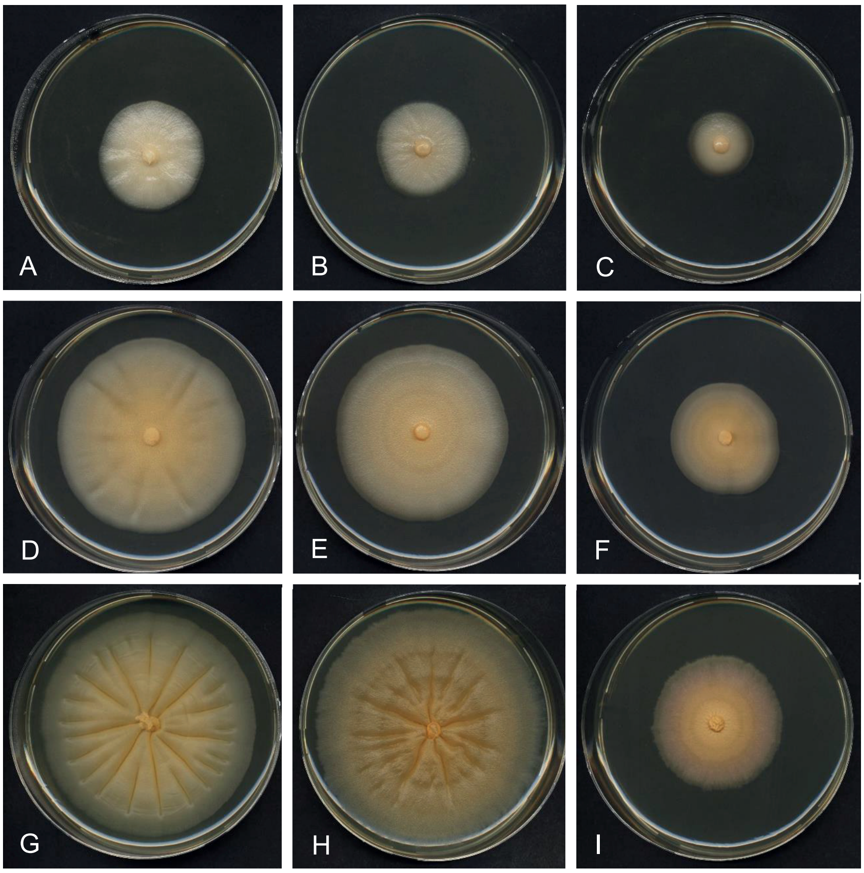

Fig. 17 *Thelebolus spongiae* MUT 2359: 21-d-old colonies on PDA at 4 °C with **A.** 0 % NaCl, **B.** 2.5 % NaCl, **C.** 5 % NaCl; at 15 °C with **D.** 0 % NaCl, **E.** 2.5 % NaCl, **F.** 5 % NaCl; at 25 °C with **G.** 0 % NaCl, **H.** 2.5 % NaCl, **I.** 5 % NaCl.

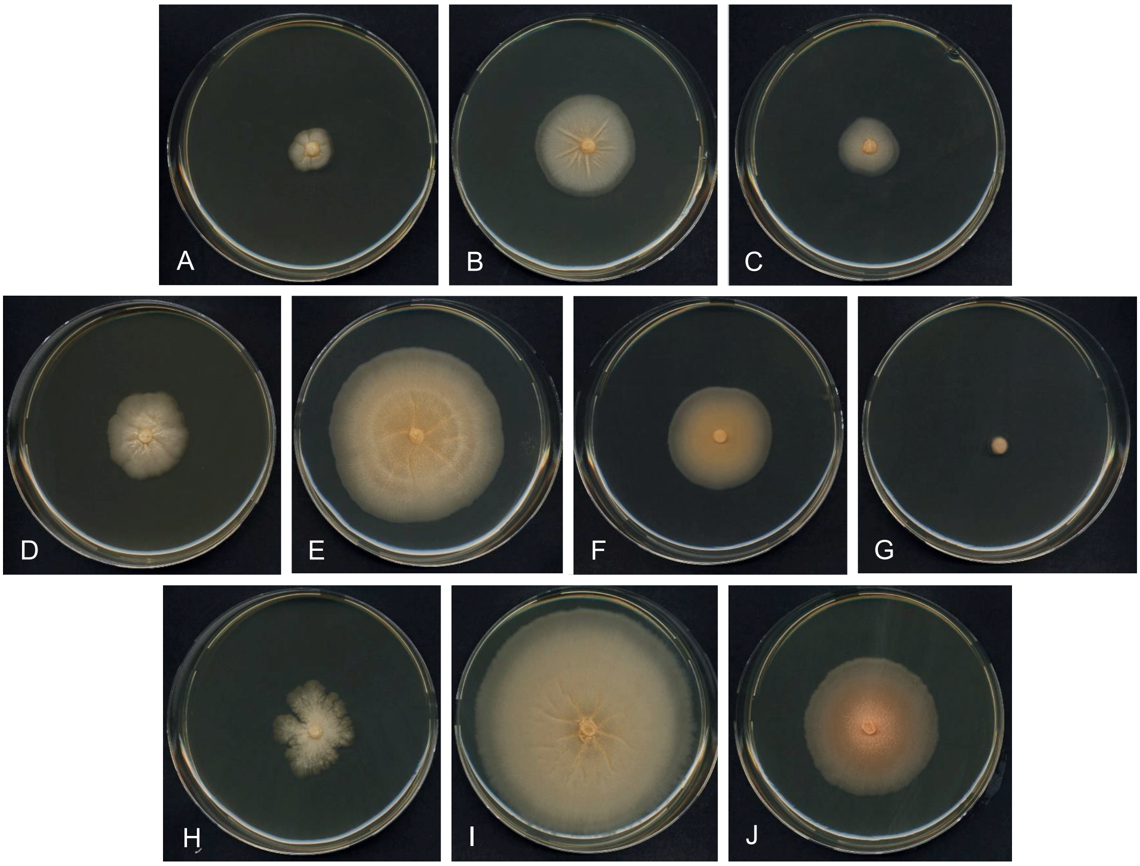

Fig. 18. *Thelebolus spongiae* MUT 2359: 21-d-old colonies on MEA at 4 °C with **A.** 0 % NaCl, **B.** 2.5 % NaCl, **C.** 5 % NaCl; at 15 °C with **D.** 0 % NaCl, **E.** 2.5 % NaCl, **F.** 5 % NaCl, **G.** 10 % NaCl; at 25 °C with **H.** 0 % NaCl, **I.** 2.5 % NaCl, **J.** 5 % NaCl.

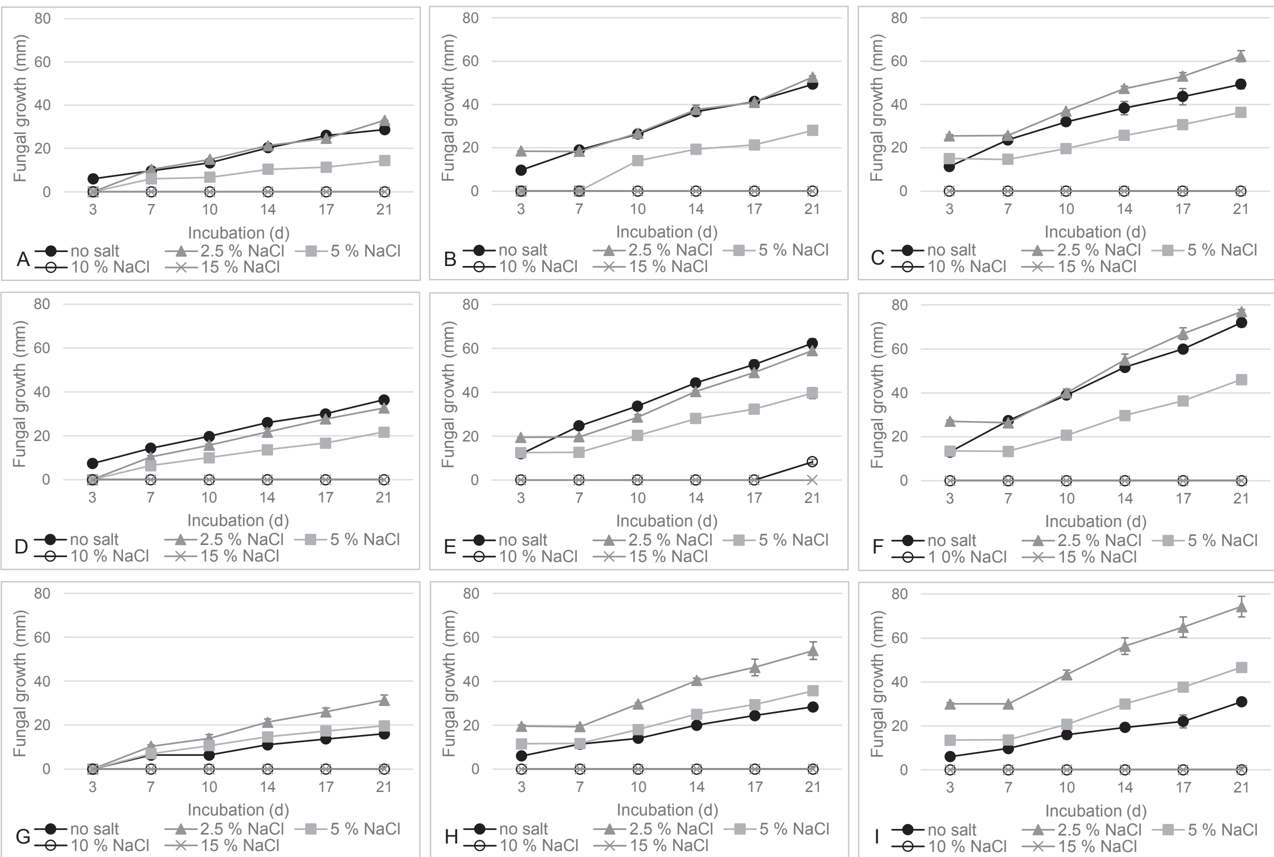

Fig. 19. *Thelebolus spongiae* MUT 2359 growth curve with no and different NaCl concentrations on CA at **A.** 4 °C, **B.** 15 °C, **C.** 25 °C; on PDA at **D.** 4 °C, **E.** 15 °C, **F.** 25 °C; on MEA at **G.** 4 °C, **H.** 15 °C, **I.** 25 °C.

Interestingly, the majority of the taxa grew exclusively at one temperature condition; this is particular evident for *P. johnstonia,* whose fungi were half isolated at 15 °C and half at 25°C.

Currently, several works on sponge-associated fungi employed different isolation techniques (Höller *et al*. 2000, Proksch *et al.* 2008, Wang *et al.* 2008, Li & Wang 2009, Ding *et al.* 2011, Wiese *et al*. 2011, Passarini *et al.* 2013, Henríquez *et al.* 2014, Diep *et al.* 2016). It would be extremely important to share these results to point out if some methods are more promising than other ones. In the attempt to increase the number of cultivable fungi, more efforts should be focused on development of innovative isolation techniques. For example, Rozas *et al.* (2011) succeeded in the isolation of fungi from single sponge cells. In parallel, the micro-Petri dishes as well as the iChip could be promising tools for the isolation of "uncultivable" (marine) microorganisms (Ingham *et al.* 2007, Nichols *et al.* 2010).

Mycobiota

Ascomycota (92 %) was the dominant phylum, as already reported for the marine environment (Jones *et al.* 2015) and for studies dealing with sponges' mycobiota (Suryanarayanan 2012). *Basidiomycota* represented a small percentage of isolates and were inferred phylogenetically by Poli *et al*. (in prep.). Their ecological role should not be underestimated: members of *Agaricales*, also detected in the present study, have already been acknowledged for their predominant role the mineralization of the organic matter in marine environment (Hyde *et al.* 1998).

Only one fungus (*Absidia glauca*) belonging to the phylum *Mucoromycota* was detected in association with *D. fragilis*. This species has already proven to withstand high salinities, as it was isolated also from Dead Sea waters. Members of *Mucoromycota* were also recorded in a small percentage in a few sponges (Höller *et al.* 2000, Thirunavukkarasu *et al*. 2012, Passarini *et al.* 2013).

According to Raghukumar (2017), sponges generally yield from zero to 21 genera of culturable fungi: while *P. johnstonia* (11 genera) is hosting an average biodiverse community, *S. ciliatum* (25) and *D. fragilis* (32) host a mycobiota communities above the mean values reported from other sponges worldwide. The most represented genera in terms of number of species were *Cladosporium* and *Penicillium* (11), followed by *Acremonium* (six) and *Aspergillus* (five). The presence of these genera and their abundance is not a surprise: they are among the most common within sponges and the most investigated for new secondary metabolites (Imhoff 2016).

Li & Wang (2009), worked on the mycobiota of three marine sponges and in the attempt to discriminate between fungi not strictly associated with sponges from those closely associated, proposed the following classification: "sponge specialist" for those genera exclusive of one sponge species, "sponge associates" for genera present in more than one species of sponge and "sponge generalist" for fungal genera present on all the species of sponges analysed. By applying this classification to the species of the present study, it was clear that the three sponges species host a specific mycobiota, as supported also by statistical analysis. For confirmation of how restricted the fungal strains are to specific sponges versus specific habitat, additional sponge species from each location should be assessed and compared to the diversity reported here.

In detail, "sponge specialist" fungi, represented more than half of the fungal community of each sponge species. Interestingly, in this group, the phylogenetic analysis highlighted several putative new species. Within the *Chaetothyriales*, MUT 2862 belongs to the genus *Cyphellophora,* which includes widespread species recorded on both animals and plants, but never described before in marine environment. This genus is in constant revision and three new species have been recently described by Gao *et al.* (2015). Within *Leotiomycetes*, MUT 2878 is well supported in the genus *Mollisia*, also known from the marine environment (Costello *et al.* 2001).

Pleosporales represent the largest group of *Dothideomycetes* and in this study, one of the most represented in terms of entities. Several fungi belong to genera already reported in the marine environment by Raghukumar (2017), this is the case of MUT 2884 (*Alternaria* sp.) MUT 2263 (*Periconia* sp.) and MUT 2390 (*Preussia* sp.). More cryptic are the position of the strains MUT 2945 (*Pleosporaceae* sp.), MUT 2489 (*Pleosporales* sp.) and MUT 2452 (*Roussoellaceae* sp.) for which further studies, are necessary. Within the *Sordariomycetes*, MUT 2766 belong to the genus *Thyronectria*, whose presence was described in the Antarctic environment by Seeler *et al*. (1940). More doubtful is the systematic classification of MUT 2463 and MUT 2377, that cluster within *Hypocreaceae* and *Microascaceae*, respectively; they both represent families already recorded in the marine environment (Jones *et al*. 2015).

Fourteen species were "sponge-associated" and common to two sponges; *D. fragilis* and *S. ciliatum* with six of these being the most similar sponges in terms of cultivable mycobiota. Among the "sponge-associated" strains, three species (*A. jensenii, A. puulaauensis* and *P. neglecta*) were reported for the first time from a marine environment. An additional seven species have never been retrieved in sponge samples but were present in the marine environment, from water samples to plants and algae samples (References details in Table 1). Two species were widespread, reported both in marine environment and associated with sponges: *B. bassiana* and *P. chrysogenum* (references in Table 2).

In the present study, the "sponge generalist" fungi were represented by *C. allicinum*, *C. cladosporioides, P. antarcticum* and *T. cylindrosporum;* all of them have been previously recorded in the marine environment and can be considered as widespread species (Bensch *et al*. 2012). *Penicillium antarcticum* is well-known both in marine (contaminated) water (Bovio *et al.* 2017) and on leaving organisms as sponges (Park *et al.* 2014) algae (Gnavi *et al*. 2017) and sea cucumbers (Marchese *et al.* 2016). *Cladosporium cladosporioides* has been reported in several marine environments, from the coral reef (Raghukumar & Ravindran 2012) to the extreme conditions of the salterns (Oren & Gunde-Cimerman 2012, Zajc *et al.* 2012) or from crude oil contaminated environments (Bovio *et al.* 2017). *Cladosporium cladosporioides* was also reported in association with marine algae (Gnavi *et al.* 2017), plants (Panno *et al.* 2013) and wood (Garzoli *et al.* 2014). Not least the presence on the sponges *Amphilectus digitata* (Pivkin *et al.* 2006), *Haliclona melana* (Rozas *et al.* 2011), *Cliona* sp. (San-Martin *et al.* 2005) and on four Red Sea sponges (Sayed *et al.* 2016). *Cladosporium allicinum* and *T. cylindrosporum* were recorded only once in the marine environment, on algae (Gnavi *et al.* 2017) and wood substrates (Rämä *et al.* 2014), respectively; while, here we documented the first report in association with marine sponges.

Considering the fact that some species, common to more than one sponge, have never been retrieved in the marine environment, it is hard to say if the classification proposed by Li & Wang (2009) is suitable to distinguish between transient mycobiota, abundant in the water columns and true sponge-associated mycobiota. This is probably due to our still scant knowledge on fungi inhabiting sea sponges and the marine environment.

The specificity of the fungal community of each sponge could be related to several factors. Pivkin *et al.* (2006) highlighted that the number of fungi associated with sponges can be influenced by the sponge structure: the harder the structure, the lower the number of fungi. Interestingly, this hypothesis is well supported in our study. *Dysidea fragilis* which has a soft structure hosted the highest number of fungal taxa (54). Actually, the sponge name is due to its fragility outside water (Marine species identification portal http://species-identification.org/index.php). *Sycon ciliatum* was the second sponge in terms of number of taxa (32) and also in a scale of body rigidity since it presents calcareous spicules although the choanocyte chambers are free from each other, giving a "loose" consistency (Marine species identification portal http://species-identification.org/index.php). *Pachymatisma johnstonia*, which hosted the lowest number of taxa (21 taxa), is characterised by the hardest structure, given by the strong cortex of up to 1 mm thickness and the presence of both macro (megascleres) and micro (microscleres) spicules.

Several other factors could be involved in sponge recruitment of specific fungi, not least the sponge bioactivity; two sponges (*D. fragilis* and *P. johnstonia*) are known for the production of bioactive metabolites, although their antifungal activity has never been demonstrated. However, the strongest proof supporting the hypothesis of the ability of the sponge to recognise and select fungi is the discovery of sponge mitochondrial introns of fungal origin and of (1→3)-β-d-glucan-binding proteins on the sponge surface for fungus recognition (Suryanarayanan 2012).

Overall, the mycobiota examined in this study was one of the most diverse compared to other sponges even from the same environment. For instance, Baker *et al.* (2009) identified 19 fungal genotypes from the sponge *Haliclona simulans* isolated in the same study area (Gurraig Sound, Co. Galway) and interestingly 85 % of the identified fungal orders were also recorded in our research. In other environments too, the biodiversity recorded was lower: seven sponges collected in the Red Sea (Egypt) yielded 22 species (Sayed *et al.* 2016); 10 Antarctic sponges hosted 24 fungal genotypes (Henríquez *et al.* 2014) while 78 taxa were isolated from six sponges of Sakhalin Island, Russia (Pivkin *et al.* 2006). Contrast the Mediterranean sponge *Psammocinia* sp. with 85 fungal taxa (Paz *et al.* 2010) and an Atlantic sponge *Dragmacidon reticulatum* with 64 taxa (Passarini *et al*. 2013).

Finally, few species reported in the present study and isolated from healthy sponges, have been previously reported as pathogenic on marine plants and animals. *Alternaria molesta* was found on a skin lesion of *Phocaena phocaena*, a marine mammal (Tóth *et al.* 2011), and was first recorded in association with a sponge in the present study. *Fusarium solani* and *Metschnikowia bicuspidata* are a threat for shrimp and prawn aquaculture (Baker *et al.* 2009, Hatai *et al.* 2012); both species have already been reported in apparently healthy sponges (Baker *et al.* 2009, Paz *et al.* 2010, Bolaños *et al.* 2015). Concerning plants, *Cladosporium perangustum* (isolated from marine water) showed pathogenic activity against mangrove leaves under laboratory conditions (Liu *et al.* 2016). These fungi are probably opportunistic pathogens, not properly able to affect healthy organisms, like the sponges of the present studies; however, further studies will be necessary to better understand their ecological role.

Two novel species of *Thelebolus*

Proving the still untapped biodiversity of marine fungi in this study it was possible to describe two new *Thelebolus* species. *Thelebolus balaustiformis* and *T. spongiae* were isolated from the Atlantic sponge *Dysidea fragilis*.

The genus *Thelebolus* has been isolated from Tropical to Artic regions, often on animal dung and from freshwater and saline lakes (de Hoog *et al.* 2005). In the marine environment, members of *Thelebolus* were recorded also associated with *Padina pavonica*, a Mediterranean brown algae (Garzoli *et al.,* in prep) and from an Antarctic marine sponge (Henríquez *et al.* 2014). In both cases, isolates were reported as *Thelebolus* sp. and the identification was based on molecular data.

Morphological characters useful to classify this genus have been long debated and, since the '70s, the number of spores per ascus represents the main character for species definition (de Hoog *et al*. 2005). At present, the genus *Thelebolus* includes 16 species and two varieties, most of which described at the end of the 19th or in the first half of the 20th century. For this reason, many of the described species, are lacking of: i) original exhaustive descriptions (i.e. microscopic characters poorly described); ii) DNA barcode sequences available in public databases; iii) ex-type strains preserved in culture collections.

Since the two *Thelebolus* species isolated in this study presented unique morphological and molecular features, we performed a deep bibliographic search to define the main characters for each described *Thelebolus* species (Table 3) and for those available in culture collections, we obtained comparable sequences, which are now available to the scientific community. The two new marine species can be easily distinguished because they form well-defined lineages within the genus (Fig. 9). Interestingly, the isolates MUT 2357 clustered with a marine strain (*Thelebolus* sp. MUT 5281), already present in MUT culture collection and isolated from a Mediterranean brown alga; this indicates the strong affinity of this species with the marine environment. From a morphological point of view, all the dichotomous keys of the genus point out as first statement the presence of 8-spored or multisporic asci (Doveri 2004, de Hoog *et al.* 2005). Therefore, considering only the multi-spored species (not included in the tree because there were no available sequences) we can first exclude the similarity of *T. balaustiformis* with *T. monoascus* and *T. pilosus*, in fact, the last two mentioned species present only one ascus per ascoma and a higher number of spores compared to MUT 2357. *Thelebolus balaustiformis* differs also from the two varieties of *T. dubius,* by presenting a higher number of asci (20–30) and a lower number (48–64) of smaller spores.

Thelebolus spongiae MUT 2359, is characterised by a variable number of asci (from one to six), while in *T. monoascus* and *T. pilosus* is strictly limited to one; the latest mentioned species differs from *T. spongiae* MUT 2359 also for the shape and size of ascospores. *Thelebolus dubius* var. *lagopi* and *Thelebolus dubius* var. *dubius* present a variable number of asci, starting from three; the shape of asci, as well as those of ascospores differ from *T. spongiae*. In fact, MUT 2359 present globular to sacciform asci and peculiar ascospores with different ratio (2.2–2.4) from *T. dubius* var. *lagopi* (1.5) and *T. dubius* var. *dubius* (1.7).

Table 3. *Thelebolus* species and main morphological features (ascomata, asci and ascospores).

Species	Ascomata	Number of asci per ascoma	Asci	Number of ascospores	Ascospores	References
T. coemansii	-	numerous	85–110 × 20–25 µm, cylindrical-clavate	8	-	(13)
T. delicatus[a]	Subglobosus	-	-	-	-	(5)
T. dubius* var. *dubius	-	3–5	40–45 × 24.4 µm, broadly ovate or oblong-ovate	128 (?)	6 × 4 µm, ellipsoid, rather pointed at the ends	(4)
T. dubius* var. *lagopi	80–150 µm diam subglobosus	10–16	87–100 × 25–33 µm, cylindrical-clavate	more than 200	6.2–7.6 × 3.6–4.3 µm, ellipsoid to ovoid	(4)
T. ellipsoideus	17–46 µm diam, subglobosus or ovoid to ellipsoid	1–8 (rarely up to 25)	22 × 11–16 µm, shortly ellipsoid to subglobose	8	5–9.2 × 4–5.3 µm, shortly-ellipsoid	(6)
T. globosus	300–520 µm diam, subglobosus or ovoid to ellipsoid	1–4	12–15 × 9–12 µm, irregular shortly ellipsoid to subglobose	8	5–7.5 × 4.1–5.1 µm, broadly ellipsoid	(6)
T. hirsutus[a]	-	-	-	-	-	(2)
T. lignicola	-	-	-	60–100	3.4 x 4–4.5 µm	(8)
T. microcarpus	18–70 µm diam, globose to subglobose	1–5	12–17 × 10–15 µm, subglobose to broadly ellipsoid	8	5–9 × 3–4 µm, ellipsoid	(1)
T. microsporus	45–500 µm diam, subglobosus, hemispheric or subcylindric	5–100	80–125 × 20–26 µm, cylindrical to cylindrical-clavate	8	6–10 × 3–5	(4), (6), (7)
T. minutissimus[a]	-	-	-	-	-	(3)
T. monoascus	150–200 µm diam, hemispheric	1	150–170 µm	500	5–6.5 × 4– 4.5 µm, ovate	(9)
T. pilosus	-	1	300 × 250 µm	about 100	9–11 × 7– 8 µm	(11)
T. polysporus	60–200 µm diam	2–5	50–160 × 18–90 µm, subellipsoid to ovoid or sacciform	256	5–7.5 × 3–4 µm, ovoid to oblong-ellipsoidal	(4), (7), (13)
T. stercoreus	135–400 µm diam, ellipsoid to ovoid or subglobose	1, rarely 2–3	165–262 × 120–205 µm, ellipsoid to ovoid or subglobose	up to 3 000 spores	5 –7.7 × 2.3–4.5 µm, spores smooth, broadly elliptic, ellipsoid or oblong	(4), (7), (13)
T. striatus	-	-	124–162 × 9–10.8 µm, elongate cylindrical	8	11.3–13.5 × 6–6.7 µm, narrow ellipsoid	(12)
T. terrestris	-	-	-	-	18.4–25.6 × 8–9.6 µm	(10)

(1) Crous *et al.* 2015, (2) De Lamarck & De Candolle 1815, (3) De Schweinitz 1834, (4) Doveri 2004, (5) Fries 1823, (6) de Hoog *et al.* 2005, (7) Kimbrough 1981, (8) Lloyd 1918, (9) Mouton 1886, (10) Pfister 1993, (11) Schroeter 1908, (12) Thind *et al.* 1959, (13) Van Brummelen 1998.

[a]The original descriptions do not contain any information about the microscopic structures.

Interestingly, *T. balaustiformis* was isolated by homogenisation of sponges tissues on CMASW at 15 °C, while *T. spongiae* was isolated by direct plating of sponge tissue on SWA at 15 °C, highlighting once more the importance of using different isolation techniques and culture conditions.

In conclusion, with the present work, we highlighted the great and still unexplored fungal diversity that characterises the marine environment. The use of several isolation methods improved the yield of cultivable fungi that with few techniques and growth media, would have been impossible to isolate. The sponges proved to host a specific mycobiota and several fungi identified with the contribution of morphological, molecular and phylogenetic approach, were first reported from a marine environment, while *T balaustiformis* and *T.*

spongiae were here described as new. The present study again highlights the great mosaic of largely unknown marine microbial diversity.

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Supplementary Table 1. List of fungal strains isolated from *D. fragilis* (DF), *P. johnstonia* (PJ) and *S. ciliatum* (SC) with Mycotheca Universitatis Taurinensis (MUT) accession number (available on: http://www.mut.unito.it/en/Database) and GenBank accession number.

MUT Accession number	Source	Taxa	GenBank accession number						
			ITS	LSU	*TUB*	*ACT*	*CAL*	*GAPDH*	D1-D2
2436	DF	*Absidia glauca*	MG813192						
2491	DF	*Acremonium breve*	MG813193						
2365	DF	*Acremonium implicatum*	MG813194						
2367	DF	*Acremonium persicinum*	MG813195						
2360	DF	*Acremonium potronii*	MG813196	MG816494					
2439	DF		MG813197	MG816495					
2462	DF		MG813198	MG816496					
2440	DF		MG813199	MG816497					
2809	SC		MG813210	MG816501					
2355	DF	*Acremonium tubakii*	MG813200						
2378	DF		MG813201						
2818	SC	*Acremonium zonatum*	MG813213						
2370	DF	*Agaricomycetes* sp.	MF098696[a]						
2260	PJ	*Alternaria molesta*	MG813166	MG816482				MG832209	
2884	SC	*Alternaria* sp.	MG813214	MG816502				MG832211	
2513	DF	*Aspergillus creber*					MG832144		
2346	PJ						MG832143		
2226	PJ	*Aspergillus flavipes*					MG832141		
2518	DF	*Aspergillus fumigatus*			MG832191				
2908	SC				MG832204				
2520	DF	*Aspergillus jensenii*					MG832145		
2237	PJ						MG832142		
2522	DF	*Aspergillus puulaauensis*					MG832146		
2441	DF	*Aureobasidium pullulans*	MG813203						

2348	PJ		MG813169						
2523	DF		MG813172						
2805	SC	*Beauveria bassiana*	MG813215						
2882	SC		MG813216						
2425	DF	*Bimuria novae-zelandiae*	MG813173	MG816486					
2492	DF	*Bjerkandera* sp.	MF140468[a]						
2445	DF	*Boeremia exigua*	MG813174	MG816487				MG832210	
2817	SC		MG813217						
2895	SC	*Cadophora luteo olivacea*	MG813218						
2485	DF		MG813204						
2524	DF	*Cladosporium aggregatocicatricatum*	MG813175			MG832112			
2525	DF					MG832113			
2528	DF					MG832114			
2241	PJ	*Cladosporium allicinum*				MG832106			
2842	SC		MG813219			MG832122			
2935	SC					MG832124			
2529	DF					MG832115			
2532	DF	*Cladosporium cladosporioides*				MG832116			
2243	PJ					MG832107			
2245	PJ					MG832108			
2246	PJ	*Cladosporium halotolerans*	MG813163			MG832109			
2940	SC					MG832125			
2533	DF	*Cladosporium perangustum*				MG832117			
2535	DF					MG832118			
2537	DF	*Cladosporium pseudocladosporioides*				MG832119			
2248	PJ					MG832110			
2932	SC					MG832126			
2579	DF	*Cladosporium psychrotolerans*				MG832120			
2583	DF	*Cladosporium subtilissimum*				MG832121			
2249	PJ	*Cladosporium subuliforme*				MG832111			
2589	DF	*Cladosporium xylophilum*				MG832122			
2893	SC	*Coniothyrium obiones*	MG813220	MG816503					

2862	SC	*Cyphellophora* sp.	MG813221	MG816504					
2459	DF	*Emericellopsis alkalina (anamorph)*	MG813205						
2351	PJ	*Emericellopsis maritima*	MG813170	MG816484					
2458	DF	*Emericellopsis pallida (anamorph)*	MG813206						
2874	SC	*Epicoccum nigrum*	MG813222						
2594	DF	*Fusarium pseudograminearum*	MG813176						
2850	SC	*Fusarium solani*	MG813223						
2783	SC	*Gremmenia infestans*	MG813224	MG816505					
2943	SC	*Holtermanniella* sp.							MF196244[a]
2463	DF	*Hypocreaceae* sp.	MG813207	MG816499					
2941	SC	*Metschnikowia bicuspidata*							MG845236
2377	DF	*Microascaceae* sp.	MG813208	MG816500					
2878	SC	*Mollisia* sp.	MG813225	MG816506					
2599	DF	*Myrothecium cinctum*	MG813177						
2956	SC	*Neocamarosporium betae*	MG813233						
2404	DF	*Neocamarosporium calvescens*	MG813179						
2806	SC	*Paraphaeosphaeria neglecta*	MG813226						
2453	DF	*Paraphaeosphaeria neglecta (anamorph)*	MG813209						
2609	DF	*Penicillium antarcticum*			MG832193				
2735	DF	*Penicillium antarcticum*			MG832192				
2250	PJ	*Penicillium antarcticum*			MG832184				
2251	PJ	*Penicillium antarcticum*			MG832185				
2926	SC	*Penicillium antarcticum*			MG832205				
2664	DF	*Penicillium brevicompactum*			MG832194				
2665	DF	*Penicillium brevicompactum*			MG832195				
2252	PJ	*Penicillium canescens*			MG832186				
2666	DF	*Penicillium chrysogenum*			MG832196				
2704	DF	*Penicillium chrysogenum*			MG832197				
2253	PJ	*Penicillium chrysogenum*			MG832187				
2254	PJ	*Penicillium chrysogenum*			MG832188				
2255	PJ	*Penicillium chrysogenum*			MG832189				
2903	SC	*Penicillium citreonigrum*			MG832206				

2705	DF	*Penicillium inflatum*			MG832198				
2710	DF	*Penicillium janczewskii*			MG832199				
2713	DF				MG832200				
2906	SC	*Penicillium roqueforti*			MG832207				
2256	PJ	*Penicillium spinulosum*	MG813164	MG816481	MG832190				
2257	PJ	*Penicillium thomii*	MG813165						
2734	DF	*Penicillium waksmanii*			MG832201				
2887	SC	*Periconia minutissima*	MG813227						
2263	PJ	*Periconia* sp.	MG813167	MG816483					
2854	SC	*Phaeosphaeria olivacea*	MG813228	MG816507					
2928	SC	*Phaeosphaeria oryzae*	MG813229	MG816508					
2482	DF	*Phaeosphaeriopsis* sp.	MG813178	MG816488					
2959	SC	*Phaeosphaeriopsis* sp.	MG813230	MG816509					
2870	SC	*Phaeosphaeriopsis* sp.	MG813231	MG816513					
3080	SC	*Phaeosphaeriopsis* sp.	MG813232	MG816510					
2945	SC	*Pleosporaceae* sp.	MG813234	MG816511					
2489	DF	*Pleosporales* sp.	MG813180	MG816489					
2386	DF	*Pochonia suchlasporia*	MG813181						
2390	DF	*Preussia* sp.	MG813182	MG816490					
2812	SC	*Pseudeurotium bakeri*	MG813235						
2352	PJ	*Pseudocercosporella* sp.	MG813171	MG816485					
2264	PJ	*Pseudozyma* sp.							MF521974[a]
2374	DF	*Pyrenochaetopsis microspora*	MG813202	MG816498					
2452	DF	*Roussoellaceae* sp.	MG813183	MG816491					
2830	SC	*Sarocladium strictum*	MG813211						
2892	SC		MG813212						
2849	SC	*Scopulariopsis brevicaulis*	MG813236						
2266	PJ	*Sporidiobolales* sp.							MF112036[a]
2357	DF	*Thelebolus balaustiformis*	MG813184	MG816492	MG832203				
2359	DF	*Thelebolus spongiae*	MG813185	MG816493	MG832202				
2766	SC	*Thyronectria* sp.	MG813237	MG816512					
2364	DF	*Tilachlidium brachiatum*	MG813186						

2484	DF	*Tolypocladium album*	MG813187							
2406	DF	*Tolypocladium cylindrosporum*	MG813188							
2447	DF		MG813189							
2267	PJ		MG813168							
2869	SC		MG813238							
2875	SC		MG813239							
2444	DF	*Trametes gibbosa*	MF098690[a]							
3263	DF		MF098691[a]							
2397	DF	*Volutella ciliata*	MG813190							
2496	DF	*Xanthothecium peruvianum*	MG813191							

[a]Gently provided by Poli *et al*. (in prep.)

Supplementary Table 2. List of taxa used for the phylogenetic analysis of *Thelebolus* spp.

Species	Strain	Substrate	GenBank accession number	
			ITS	***TUB***
Aureobasidium pullulans	CBS 584.75[NT]	*Vitis vinifera*	KT693733	FJ157869
Sclerotinia nivalis	KACC 45150	*Aralia elata*	HM746664	JX296012
Thelebolus ellipsoideus	CBS 113937[T]	Algal mat under perennial ice in lake	AY957550	AY957542
Thelebolus globosus	CBS 113940[T]	Algal mat in lake	DQ028268	AY957547
Thelebolus microcarpus	CBS 137501	Soil	LN609269	LN609270
Thelebolus microsporus	CBS 113943	*Catharacta antarctica*	AY957554	AY957546
	CBS 109909	Algal mat in lake	AY957551	AY957543
	MUT 854	Artic soil	MG196311	MG195907
	MUT 859	Artic soil	MG196312	MG195908
	MUT 861	Artic soil	MG196313	MG195909
Thelebolus polysporus	CBS 219.69	Dung of sheep	MG196314	MG195910
Thelebolus stercoreus	CBS 375.58	Forest soil	MG196315	MG195911
Thelebolus sp.	MUT 5281	*Padina pavonica*	KT715729	MG195912