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.

**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 (Rodriguez-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 remains 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 Gurrag 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 53°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 cm³ 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 2 g, up to 1 L dH₂O) and Corn Meal Agar Sea Water – CMASW (Corn Meal 2 g, Agar 15 g, Sea Salts 30 g, up to 1 L dH₂O). 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 dH₂O), 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 morphophysiological 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 dH₂O) 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 MgCl₂ (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 sequenceda | Primers (Forward and Reverse) | PCR amplification Conditions | References for primersb |
|--------------------------------------------|--------------------------------------|--------------------------------|------------------------------|-------------------------|
| Alternaria                                 | GAPDH                                | GPD1 and GPD2                  | 96 °C: 2 min, (96 °C: 1 min, 50 °C: 1 min, 72 °C: 5 min) × 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, Pseudoszyma, 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. 1996, (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).

| Fungi                                      | RA % | 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) |    |
| Acremonium potronii                       | 5.8  | 1.0|    |
| Acremonium tubakii                         | 5.8  | (1), (34) | FR |
| Acremonium zonatum                         | 2.9  | FR |    |
| Alternaria molesta                         | 1.4  | (30) | FR |
| Alternaria sp.a                            | 1.0  | -  | -  |
| Aspergillus creber                         | 4.5  | 2.7| FR |
| Aspergillus flavipes                        | 5.4  | (22)| (35) |
| Aspergillus fumigatus                      | 2.6  | 1.0| (1), (4), (5), (7), (19) | (6), (18), (27) |
| Aspergillus jensenni                       | 0.6  | 2.7| FR |    |
| Aspergillus paulaauensis                   | 3.8  | 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 nova-zelandiae                      | 0.6  | (31) | | FR |
| Boeremia exigua                            | 0.6  | (32), (34) | FR |
| Botrytis cinerea                           | 0.6  | (5), (24), (34) | FR |
| Cadophora luteo-olivacea                   | 0.6  | 4.9| (11) | FR |
| Cladosporium aggregatocatricatum           | 0.6  | FR |    |
| Cladosporium allicinum                     | 2.6  | 3.9| (2) | FR |
| Cladosporium cladosporoides                | 2.6  | 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 |
| **Cladosporium xylophilum** | 0.6 | FR | | | FR |
| Coniothyrium obiones* | 1.0 | (7) | | | FR |
| Cyphellophora sp.* | 1.0 | - | | - | - |
| **Emericellopsis alkalina** (asexual morph) | 1.3 | | | | 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 |
| **Fusarium solani** | 1.0 | (26) | | (3), (41) | |
| Gremmenia infestans* | 0.6 | | | | FR |
| Hypocreaceae sp.* | 0.6 | - | | - | - |
| Metschnikowia bicuspidata | 5.9 | (7), (22) | | (38) | |
| Microascaceae sp.* | 0.6 | - | | - | - |
| Mollisia sp.* | 1.0 | - | | - | - |
| Myrothecium cinctum* | 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.* | 1.4 | - | | - | - |
| Phaeosphaeria olivacea* | 1.0 | (7) | | | FR |
| Phaeosphaeria oryzae* | 1.0 | FR | | | FR |
| Phaeosphaeriopsis sp.* | 0.6 | 4.9 | - | | - |
| Pleosporales sp.* | 1.0 | - | | - | - |
| Pleosporaceae sp.* | 0.6 | - | | - | - |
| Pochonia suchlasporia | 0.6 | (34) | | | FR |
| Preussia sp.* | 0.6 | - | | - | - |
| Pseudocercosporella sp.* | 1.4 | - | | - | - |
| Pyrenochaetopsis microspora* | 1.9 | FR | | | FR |
Table 2. (Continued).

| Species/Strain/Species | RA %   | DF | PJ | SY | MA | SP |
|------------------------|--------|----|----|----|----|----|
| Rousselloaceae sp.*    | 0.6    | -  | -  | -  | -  | -  |
| Sarocladium strictum   | 14.7   | (2), (7), (34) | FR |
| Scopulariopsis breviscaulis | 1.0  | (5) | (6), (13) |
| Thelebolus balastiformis| 0.6    | FR | FR | FR |
| Thelebolus spongiae    | 0.6    | FR | FR | FR |
| Thyronectria sp.*      | 1.0    | -  | -  | -  | -  |
| Tilachlidium brachiatum| 0.6    | (28) | FR |
| Tolypocladium album    | 1.9    | FR | 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 | FR |

**Basidiomycota**

- Bjerkandera sp.* 0.6 - -
- Holtermanniella sp.* 1.0 - -
- Agarycomycetes sp.* 0.6 - -
- Pseudozyma sp.* 1.4 - -
- Sporidiobolales sp.* 1.4 - -
- Trametes gibbosa* 1.3 FR FR

**Mucoromycota**

- Absidia glauca 0.6 (19) FR

Total taxa

| RA % | DF | PJ | SY | MA | SP |
|------|----|----|----|----|----|
| 54   | 21 | 32 |

Total exclusive taxa

| RA % | DF | PJ | SY | MA | SP |
|------|----|----|----|----|----|
| 39   | 11 | 21 |

*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 dH2O) 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 dH2O) 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 mycobiotics 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*)
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 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 pseudocladosporioide*
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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 Roussellaceae/Thyridariaceae. One fungus was included in the Pleosporales order. Branch numbers indicate BPP values. i.s. = incertae sedis.
Montagnulaceae

Periconiaceae

Roussoellaceae/Thyridariaceae

Sporormiaceae

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.
Microascaceae

Amphisphaeriaceae

Graphiaceae

Halosphaeriaceae

Hyponectriaceae

Lubwothiaceae, Lubwothiales

Outgroup (Pleosporales, Dothideomycetes)

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* pale yellow 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. 14A–C; the morphologies in Fig. 11. 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. balaustriformis* 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 balaustriformis* 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 MB8224103. 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 “apotheiodic” 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 globlar 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 first 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 gelatin-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 (Cabaliero-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 \textit{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 \textit{et al.} 2000, Proksch \textit{et al.} 2008, Wang \textit{et al.} 2008, Li & Wang 2009, Ding \textit{et al.} 2011, Wiese \textit{et al.} 2011, Passarini \textit{et al.} 2013, Henríquez \textit{et al.} 2014, Diep \textit{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 \textit{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 \textit{et al.} 2007, Nichols \textit{et al.} 2010).

**Mycobiota**

\textit{Ascomycota} (92 \%) was the dominant phylum, as already reported for the marine environment (Jones \textit{et al.} 2015) and for studies dealing with sponges’ mycobiota (Suryanarayanan 2012). \textit{Basidiomycota} represented a small percentage of isolates and were inferred phylogenetically by Poli \textit{et al.} (in prep.). Their ecological role should not be underestimated: members of \textit{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 \textit{et al.} 1998).

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

According to Raghukumar (2017), sponges generally yield from zero to 21 genera of culturable fungi: while \textit{P. johnstonia} (11 genera) is hosting an average biodiversity community, \textit{S. ciliatum} (25) and \textit{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 \textit{Cladosporium} and \textit{Penicillium} (11), followed by \textit{Acremonium} (six) and \textit{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: “spoon specialist” for those genera exclusive of one sponge species, “spoon associates” for genera present in more than one species of sponge and “spoon 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, “spoon 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 \textit{Chaetothyriales}, MUT 2862 belongs to the genus \textit{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 \textit{et al.} (2015). Within \textit{Leotiomycetes}, MUT 2878 is well supported in the genus \textit{Mollisia}, also known from the marine environment (Costello \textit{et al.} 2001).

\textit{Pleosporales} represent the largest group of \textit{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 (\textit{Alternaria} sp.) MUT 2263 (\textit{Periconia} sp.) and MUT 2390 (\textit{Preussia} sp.). More cryptic are the position of the strains MUT 2945 (\textit{Pleosporaeeae} sp.), MUT 2489 (\textit{Pleosporales} sp.) and MUT 2452 (\textit{Roussellae} sp.) for which further studies, are necessary. Within the \textit{Sordariomycetes}, MUT 2766 belong to the genus \textit{Thyronectria}, whose presence was described in the Antarctic environment by Seeler \textit{et al.} (1940). More doubtful is the systematic classification of MUT 2463 and MUT 2377, that cluster within \textit{Hypocreaceae} and \textit{Microasceaceae}, respectively; they both represent families already recorded in the marine environment (Jones \textit{et al.} 2015).

Fourteen species were “sponge-associated” and common to two sponges; \textit{D. fragilis} and \textit{S. ciliatum} with six of these being the most similar sponges in terms of cultivable mycobiota. Among the “sponge-associated” strains, three species (\textit{A. jensenii}, \textit{A. puulauensis} and \textit{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: \textit{B. bassiana} and \textit{P. chrysogenum} (references in Table 2).

In the present study, the “sponge generalist” fungi were represented by \textit{C. allicinum}, \textit{C. cladosporioides}, \textit{P. antarcticum} and \textit{T. cylindrosporum}; all of them have been previously recorded in the marine environment and can be considered as widespread species (Bensch \textit{et al.} 2012). \textit{Penicillium antarcticum} is well-known both in marine (contaminated) water (Bovio \textit{et al.} 2017) and on leaving organisms as sponges (Park \textit{et al.} 2014) algae (Gnavi \textit{et al.} 2017) and sea cucumbers (Marchese \textit{et al.} 2016). \textit{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, Jazc \textit{et al.} 2012) or from crude oil contaminated environments (Bovio \textit{et al.} 2017). \textit{Cladosporium cladosporioides} was also reported in association with marine algae (Gnavi \textit{et al.} 2017), plants (Panno \textit{et al.} 2013) and wood (Garzoli \textit{et al.} 2014). Not least the presence on the sponges \textit{Amphilectus digitata} (Pivkin \textit{et al.} 2006), \textit{Halictothrix melana} (Rozas \textit{et al.} 2011), \textit{Cliona} sp. (San-Martin \textit{et al.} 2005) and on four Red Sea sponges (Sayed \textit{et al.} 2016). \textit{Cladosporium allicinum} and \textit{T. cylindrosporum} were recorded only once in the marine environment, on algae (Gnavi \textit{et al.} 2017) and wood substrates (Rámá \textit{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 (megascles) and micro (microscles) 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 (Gurraga 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 Psamnocinia 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).
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 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.

### 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*   | Subglobosus            | -                         | 40–45 × 24.4 µm, broadly ovate or oblong-ovate | 128 (?)              | 6 × 4 µm, ellipsoid, rather pointed at the ends | (4)       |
| *T. dubius var. dubius* | 80–150 µm diam | 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. dubius var. lagopi* | 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. ellipoideus* | 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*    | -                      | -                         | -                                | -                    | -                           | (2)       |
| *T. lignicola*   | -                      | -                         | 60–100                           | 3.4 × 4–4.5 µm       | (8)                         |           |
| *T. microcarpus* | -                      | -                         | 18–70 µm diam, globose to subglobose | 1–5                  | 5–9 × 3–4 µm, ellipsoid     | (1)       |
| *T. microsporus* | -                      | -                         | 45–500 µm diam, subglobosus, hemispheric or subcylindric | 5–100                | 6–10 × 3–5                  | (4), (6), (7) |
| *T. minutissimus* | -                      | -                         | -                                | -                    | -                           | (3)       |
| *T. monoascus*   | -                      | -                         | 150–200 µm diam, hemispheric      | 150–170 µm           | 5–6.5 × 4–4.5 µm, ovate     | (9)       |
| *T. pilosus*     | -                      | -                         | 60–200 µm diam                   | 1                   | 300 × 250 µm, about 100     | (11)      |
| *T. polysporus*  | -                      | -                         | 6–10 × 3–5                      | (4), (7), (13)      |                             |           |
| *T. stercoreus*  | -                      | -                         | 135–400 µm diam, ellipsoid to ovoid or subglobose | 1, rarely 2–3       | 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 Lamarrck & 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.

*The original descriptions do not contain any information about the microscopic structures.*
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 |
|----------------------|--------|------|
|                      |        |      |
|                      |        |      |
| 2436                 | DF     | *Absidia glauca* |
| 2491                 | DF     | *Acremonium breve* |
| 2365                 | DF     | *Acremonium implicatum* |
| 2367                 | DF     | *Acremonium persicinum* |
| 2360                 | DF     | *Acremonium potronii* |
| 2439                 | DF     |        |
| 2462                 | DF     |        |
| 2440                 | DF     |        |
| 2809                 | SC     |        |
| 2355                 | DF     | *Acremonium tubakii* |
| 2378                 | DF     |        |
| 2818                 | SC     | *Acremonium zonatum* |
| 2370                 | DF     | *Agaricomycetes sp.* |
| 2260                 | PJ     | *Alternaria m offshore* |
| 2884                 | SC     | *Alternaria sp.* |
| 2513                 | DF     | *Aspergillus creber* |
| 2346                 | PJ     |        |
| 2226                 | PJ     | *Aspergillus flavipes* |
| 2518                 | DF     | *Aspergillus fumigatus* |
| 2908                 | SC     |        |
| 2520                 | DF     | *Aspergillus jenanii* |
| 2237                 | PJ     |        |
| 2522                 | DF     | *Aspergillus puulauensis* |
| 2441                 | DF     | *Aureobasidium pullulans* |

| ITS    | LSU | TUB | ACT | CAL | GAPDH | D1-D2 |
|--------|-----|-----|-----|-----|-------|-------|
|        |     |     |     |     |       |       |
|        |     |     |     |     |       |       |
|        |     |     |     |     |       |       |
| MG813192 |     |     |     |     |       |       |
| MG813193 |     |     |     |     |       |       |
| MG813194 |     |     |     |     |       |       |
| MG813195 |     |     |     |     |       |       |
| MG813196 | MG816494 |     |     |     |       |       |
| MG813197 | MG816495 |     |     |     |       |       |
| MG813198 | MG816496 |     |     |     |       |       |
| MG813199 | MG816497 |     |     |     |       |       |
| MG813200 |     |     |     |     |       |       |
| MG813201 |     |     |     |     |       |       |
| MG813213 |     |     |     |     |       |       |
| MG098696* |     |     |     |     |       |       |
| MG813166 | MG816482 |     |     |     |       | MG832209 |
| MG813214 | MG816502 |     |     |     |       | MG832211 |
| MG832144 |     |     |     |     |       |       |
| MG832143 |     |     |     |     |       |       |
| MG832141 |     |     |     |     |       |       |
| MG832191 |     |     |     |     |       |       |
| MG832204 |     |     |     |     |       |       |
| MG832145 |     |     |     |     |       |       |
| MG832142 |     |     |     |     |       |       |
| MG832146 |     |     |     |     |       |       |
| MG813203 |     |     |     |     |       |       |
|   |   |   |   |
|---|---|---|---|
|   |   |   |   |
|   |   |   |   |
|   |   |   |   |
|   |   |   |   |
| 2348 | PJ |   | MG813169 |
| 2523 | DF |   | MG813172 |
| 2805 | SC |   | MG813215 |
| 2882 | SC |   | MG813216 |
| 2425 | DF |   | MG813173 MG816486 |
| 2492 | DF |   | MG813177 |
| 2445 | DF |   | MG813217 |
| 2817 | SC |   | MG813218 |
| 2895 | SC |   | MG813219 |
| 2485 | DF |   | MG813220 |
| 2524 | DF |   | MG813217 MG832112 |
| 2525 | DF |   | MG832113 |
| 2528 | DF |   | MG832114 |
| 2241 | PJ |   | MG832106 |
| 2842 | SC |   | MG832115 |
| 2935 | SC |   | MG832116 |
| 2529 | DF |   | MG832107 |
| 2532 | DF |   | MG832108 |
| 2243 | PJ |   | MG832107 |
| 2246 | PJ |   | MG832109 |
| 2940 | SC |   | MG832125 |
| 2533 | DF |   | MG832117 |
| 2535 | DF |   | MG832118 |
| 2537 | DF |   | MG832119 |
| 2248 | PJ |   | MG832120 |
| 2932 | SC |   | MG832126 |
| 2579 | DF |   | MG832120 |
| 2583 | DF |   | MG832121 |
| 2249 | PJ |   | MG832111 |
| 2589 | DF |   | MG832122 |
| 2893 | SC |   | MG83220 MG816503 |
| Number | Type | Species Name | Accession Numbers |
|--------|------|--------------|-------------------|
| 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* |
| 2463   | DF   | Hypocreaceae sp. | MG813207 MG816499 |
| 2941   | SC   | Metschnikowia bicuspidata | MG845236 |
| 2377   | DF   | Microasaceae 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 brevicompactum | MG832192 |
| 2250   | PJ   | Penicillus canescens | MG832184 |
| 2251   | PJ   | Penicillium chrysogenum | MG832185 |
| 2926   | SC   | Penicillium citreonigrum | MG832205 |
| 2664   | DF   | Penicillium chrysogenum | MG832194 |
| 2665   | DF   | Penicillium chrysogenum | MG832195 |
| 2252   | PJ   | Penicillium canescens | MG832186 |
| 2666   | DF   | Penicillium citreonigrum | MG832196 |
| 2704   | DF   | Penicillium chrysogenum | MG832197 |
| 2253   | PJ   | Penicillium canescens | MG832187 |
| 2254   | PJ   | Penicillium chrysogenum | MG832188 |
| 2255   | PJ   | Penicillium canescens | MG832189 |
| 2903   | SC   | Penicillium citreonigrum | MG832206 |
|   |   | Species                                      | Accession Numbers         |
|---|---|---------------------------------------------|---------------------------|
|2705 | DF | *Penicillium inflatum*                      | MG832198                  |
|2710 | DF | *Penicillium janczewskii*                   | MG832199                  |
|2713 | DF |                                           |                           |
|2906 | SC | *Penicillium roqueforti*                    | MG832207                  |
|2256 | PJ | *Penicillium spinulosum*                    | MG813164, MG816481, MG832190 |
|2257 | PJ | *Penicillium thomii*                        | MG813165                  |
|2734 | DF | *Penicillium waksmanii*                     |                           |
|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 | *Pseudocercospora sp.*                      | MG813171, MG816485        |
|2264 | PJ | *Pseudozyma sp.*                            |                           |
|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.*                       |                           |
|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                  |
### Supplementary Table 2. List of taxa used for the phylogenetic analysis of *Thelebolus* spp.

| Species                        | Strain      | Substrate                   | GenBank accession number |
|-------------------------------|-------------|-----------------------------|--------------------------|
| *Aureobasidium pullulans*     | CBS 584.75NT| *Vitis vinifera*            | KT693733                 |
| *Sclerotinia niva*            | KACC 45150  | *Aralia elata*              | HM746664                 |
| *Thelebolus ellipsoideus*     | CBS 113937† | Algal mat under perennial ice in lake | AY957550     |
| *Thelebolus globosus*         | CBS 113940‡ | Algal mat in lake           | DQ028268                 |
| *Thelebolus microcarpus*      | CBS 137501  | Soil                        | LN609269                 |
|                               | CBS 113943  | *Catharacta antarctica*     | AY957554                 |
| *Thelebolus microsporus*      | CBS 109909  | Algal mat in lake           | AY957551                 |
|                               | MUT 854     | Artic soil                  | MG196311                 |
|                               | MUT 859     | Artic soil                  | MG196312                 |
|                               | MUT 861     | Artic soil                  | MG196313                 |
| *Thelebolus polysporus*       | CBS 219.69  | Dung of sheep               | MG196314                 |
| *Thelebolus stercoreus*       | CBS 375.58  | Forest soil                 | MG196315                 |
| *Thelebolus sp.*              | MUT 5281    | *Padina pavonica*           | KT715729                 |

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