Diversity and bioactivity of fungi associated with the marine sea cucumber Holothuria poli: disclosing the strains potential for biomedical applications

Diversity and bioactivity of fungi associated with the marine sea cucumber Holothuria poli*: disclosing the strains potential for biomedical applications*

Pietro Marchese,[a,b] Laura Garzoli[b], Giorgio Gnavi[b], Enda O'Connell[c], Abderrahman Bouraoui[d], Mohamed Mehiri[e], Mary Murphy[a], Giovanna Cristina Varese[b]

[a]National University of Ireland Galway, Regenerative Medicine Institute, School of Medicine, Galway, Ireland.

[b]University of Turin, *Mycotheca Universitatis Taurinensis,* Department of Life Sciences and Systems Biology, Turin, Italy.

[c]National University of Ireland Galway, Genomics and Screening Core, Galway, Ireland

[d]Universite de Monastir, Laboratoire de dévelopement chimique, galénique et pharmacologique des médicaments, Monastir, Tunisia.

[e]University Nice Sophia Antipolis, Marine Natural Products Team, Institute of Chemistry of Nice, Nice, France.

Address correspondence to: Pietro Marchese, Regenerative Medicine Institute, National University of Ireland, Biosciences Building, Newcastle Road, Dangan, H91TK33, Galway, Ireland email:p.marchese1@nuigalway.ie, tel. +353 091495206

Running head: Bioactive fungi of *Holothuria poli*

Abstract

Aims: Identification of the mycobiota associated to the marine echinoderm *Holothuria poli* and investigation of cytotoxic and pro-osteogenic potential of isolated strains.

Methods and results: Fungal strains were isolated from the animals body-wall, intestine, and faeces. The species identification was based on DNA barcoding and morphophysiological observations. Forty-seven species were identified, all Ascomycota and mainly belonging to *Aspergillus* and *Penicillium* genera. Sixteen strains were grown on three media for chemical extraction. Cytotoxic activity was tested on a hepatic cancer cell line (HepG2), the cells viability was evaluated after treatment using a resazurin based assay (AlamarBlue). Pro-osteogenic activity was tested on human Mesenchymal Stem Cell, differentiation was measured as the alkaline phosphatase production through reaction with *p*-nitrophenylphosphate or as the cells ability to mineralize calcium using a colorimetric kit (StanBio). Cytotoxic activity was recorded for four fungal species while five out of 48 extracts highlighted bioactivity toward human mesenchymal stem cells.

Conclusions: The presence of relevant animal-associated mycobiota was observed in *H. poli* and selected strains showed cytotoxic potential and pro-osteogenic activity.

Significance and importance of this study: Our work represents the first report of a Mediterranean sea cucumber mycobiota and highlights the isolates potential to synthetize compounds of pharmaceutical interest for regenerative medicine.

Keywords: Environmental mycology, Pharmaceuticals, Cytotoxicity, Marine Fungi, Sea cucumber, Mediterranean Sea

Introduction

Fungi are important actors of the marine ecosystem, studies on the molecular diversity of the micro-eukaryotic community shown that they inhabit most of the marine habitats with disparate ecological roles (Jones and Pang 2012). To date, 1,412 species have been documented in the marine environment (Jones et al. 2019) but information on unexplored habitats and DNA sequencing data collected over the past 20 years identified a possible further 10,000 (Jones 2011). To overcome this lack of knowledge and encrypt the real microbial biodiversity existing in the sea, the isolation and identification of fungal communities living in association with new substrates is an essential task. Several studies investigated the mycobiota existing in the Mediterranean Sea, mainly focusing on algae, sponges and seagrasses (Garzoli *et al.* 2014; Gnavi *et al.* 2017; Bovio *et al.* 2019), describing particular marine fungal communities living in association with marine organisms. The up to date picture shows over 200 fungal species isolated from Mediterranean substrates, with several recurring taxa such as *Aspergillus*, *Penicillium* and *Acremonium*. Common species such as *Penicillium chrysogenum*, *Trichoderma harzianum* and *Cladosporium sphaerospermum* were also identified, but the discovery of key species and trends in marine fungal populations strongly rely on the description of fungal communities from additional substrates.

Beside their ecological value, the description and isolation in pure culture of fungal strains from new marine substrates represent a valuable resource for biotechnological applications for their potentially unusual biochemical properties. Marine derived fungi had shown potential to synthetize pharmaceutical compounds such as anticancer, antibacterial, anti-inflammatory, antiviral, pro-osteogenic and others (Ebel 2012; Prince and Samuel 2015; Silber *et al.* 2016) as well as compounds with cosmeceutical and nutraceutical properties (Imhoff 2016). Marine drug discovery surveys had underestimated the microbial potential in the past, often focusing on bioactive molecules extracted from macro-organism without considering the substrate microbial colonization. This approach led to mistakenly assign the biosynthesis of chemotherapeutic Ectainascidin to the marine tunicate *Ecteinascidia turbinata* (Rinehart *et al.* 1990) instead of the real producer, its bacterial endosymbiont (Schofield *et al.* 2015). To address the microbe as the metabolite producer instead of an animal or plant is very advantageous: once isolated, a fungus can be grown *in vitro*, allowing further studies with no more expensive and environmental-impacting sampling campaigns. Moreover, the development of fermenters for fungal cultivation allows industrial production and extraction of the metabolite from the biomass (Syed 2019).

Holothuria poli (Delle Chiaje, 1823) is a Mediterranean and oceanic sea cucumber, widely distributed in the Mediterranean Sea as well as in the northern Red Sea and in the Canary Islands Sea (WORMS 2020). It has been intensively studied for its secondary metabolites production, demonstrating a strong antifungal activity (Ismail *et al.* 2008), but never deeply analysed for its associated microbiome. A single study by Omran and Allam (2013) isolated one

97 strain of *Candida albicans* and a few bacteria associated with specimens
98 collected in Egyptian Mediterranean sea, while a rich mycobiota was recorded
99 by Pivkin (2000) on internal and external organs of three species of sea
100 cucumbers sampled in the Pacific Ocean. A thorough study of the mycobiota
101 associated to *H. poli* is particularly important for the wide distribution of this
102 echinoderm in the Mediterranean area and for its ecological role as filter feeder.
103 Given its remarkable production of antifungal compounds, the associated
104 mycobiota might be specifically selected and contain strains with particular
105 biochemical properties and potential producers of new pharmaceutically
106 bioactive compounds.

107 In this study we describe the fungal community living in association with
108 *H. poli*. The isolates biotechnological potential was investigated as their ability
109 to synthetize metabolites for pharmaceutical applications. Investigated targets
110 were the cytotoxicity against an hepatic cancer cell line (HepG2) and the
111 bioactivity toward stem cell to promote their differentiation into bone or cartilage
112 cell progenitors (Alves *et al.* 2011; Besio *et al.* 2019a, 2019b).

113

114 Materials and methods

115 *Holoturia poli* collection

116 Six individuals of *H. poli*, together with their faeces (rejected sand), were
117 collected in September 2013 along the rocky coast of the Tabarka peninsula
118 (Tunisia). Samples were maintained at 0-4°C during transportation. In order to
119 evaluate the fungal colonization on different animal districts, specimens were
120 firstly washed in sterile seawater, surface sterilized with 70% ethanol and then

underwent surgical manipulation in a sterile condition to separate the body wall (B), intestine (I) and faeces (S).

Isolation and identification of associated fungal strains

Each sample was homogenized using steel beads and MM400 tissue lyzer (Retsch GmbH, Haan, Germany), then was diluted 1:10 w/v in phosphate buffer. An aliquot of each sample was dried to calculate the samples' number of colony forming units *per* gram of dry weight (CFU gdw^{-1}). One ml of suspension was plated on Corn Meal Agar Sea Water (CMASW: 2g corn meal extract, 15g agar dissolved in 1l of sterilized artificial seawater, 2% w/v Sea Salts in ddH_2O) added of an antibiotic mix (Gentamicin 80mg l^{-1}, Piperacillin-Tazobactam 100mg l^{-1} - Sigma-Aldrich, Saint Louis, USA). Three replicates *per* sample were performed (Panno *et al.* 2013; Gnavi *et al.* 2017). A total of 54 plates were incubated at 24°C in the dark up to one month to allow the isolation of all fungi, including the slow-growing ones. Plates at days 2, 7, 14, and 21. Strains from each fungal morphotype and from each matrix (B, I, S) were isolated in axenic culture and preserved at the *Mycotheca Universitatis Taurinensis* (MUT – http://www.mut.unito.it/en).

Identification of the isolated fungi was carried out with a polyphasic approach, combining morpho-physiological and molecular methods. Fungi were firstly morphologically classified on the basis of specific taxonomical keys (Seifert *et al.* 2011; Samson *et al.* 2014a, 2014b). Subsequently, molecular analyses were performed by sequencing specific genomic regions (for details see Table S1). Taxonomic assignments were based on similarity to reference sequences

145 available at GenBank (nBlast; mismatch 1/-2; gap costs linear) and CBS
146 databases. Molecular findings were confirmed morpho-physiologically. Newly
147 generated sequences were deposited in GenBank database (Table S2)

148

149 **Fungal chemical extraction**

150 Sixteen strains (Tab. 3), encompassing the most representative species
151 isolated from *H. poli*, were cultivated following a preliminary OSMAC (One
152 Strain Many Compounds – Bode *et al.*, 2002) approach. Fungi were cultured
153 on different nutrient conditions to stimulate the production of different
154 metabolites: each strain was inoculated in duplicate (two 9cm Petri dishes for
155 each condition), onto three media containing different carbon and nitrogen
156 sources and/or salinity. Based on pre-trials, media used were: Malt Extract Agar
157 (MEA: agar 20g, glucose 20g, malt extract 20g, peptone 2g, dissolved in 1l
158 ddH_2O), Malt Extract Chloride Agar (MEClA: agar 20g, malt extract 20g, NaCl
159 17g, dissolved in 1l ddH_2O) and Soy Mannitol Agar (SMA: agar 20g, soy
160 peptone 20g, mannitol 20g, NaCl 15g, dissolved in 1l ddH_2O). Plates were
161 incubated at 24°C for four weeks to allow fungal growth. After incubation, fungal
162 biomass and medium were lyophilized and minced using an Ultraturrax device
163 (IKA, Staufen, Germany), extracted adding 1:10 w/v of solvent mix (1:1
164 methanol–dichloromethane) and stirred overnight. Suspensions were filtered
165 and dried using Rotavapor (KNF, Freiburg, Germany). Using this procedure 48
166 crude dry extracts were obtained.

167

168 **Fungal extracts chemical fingerprinting**

Extracts chemical fingerprint was performed by analytical HPLC with a Waters Alliance 2695 (Waters Corporation, Milford, MA) using Luna C18 column (250×10mm id, 5µm Phenomenex Inc., Torrance, USA) coupled to a Diode Array Detector (DAD, Waters 996). The crude extracts were re-suspended at final concentrations of 10mg ml^{-1} in CH_2Cl_2/MeOH (1:1, v/v), 20µl were injected and eluted with a gradient of H_2O/MeCN/Formic acid (90:10:0.1 to 0:100:0.1 – flow 1ml min^{-1}). Detection was performed by Sedex 55 evaporative light-scattering detector (SEDERE, Alfortville, France), wavelengths set at 214, 254 and 280nm.

Extracts cytotoxicity assessment

To assess the extracts cytotoxicity, a miniaturized high throughput assay was performed using an automated workstation (PerkinElmer, Waltham, USA). A liver cancer cell line (HepG2) was treated with seven extracts concentrations (200pg l^{-1}, 2ng ml^{-1}, 20ng ml^{-1}, 200ng ml^{-1}, 2µg ml^{-1}, 20µg ml^{-1} and 200µg ml^{-1}): dry extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, USA) and diluted in cell culture medium to reach the working concentrations.

Cells were cultured in Basic Medium (BM) containing: αMEM and 1% penicillin/streptomycin - Thermofisher, Waltham, USA plus 10% FBS – Sigma Aldrich, Saint Louis, USA. Culture flasks were maintained at 37°C, 5% CO_2 in humidified atmosphere. The screening was performed by seeding $5x10^3$ cells in in each well of flat-bottom 96-well plates, incubated to settle overnight. Subsequently, treatment was performed by adding 100µl of BM with either

diluted extracts or controls, all conditions tested in triplicate. DMSO was kept at 0.5% in the experimental wells. The positive control cells were treated with 0.5% DMSO and negative controls with 10% DMSO to induce a cytotoxicity. Cells were then incubated at 37°C, 5% CO_2 for 72 h.

Cell viability after treatment was evaluated by measuring the cell reduction of the vital reagent resazurin (AlamarBlue - Thermofisher, Waltham, USA). A 10% v/v of reagent was added to the wells and plates incubated for 6 h at 37°C, 5% CO_2 to let the cells metabolize the reagent. After incubation, fluorescence was measured at 531nm excitation wavelength, 572nm emission wavelength, and Lethal Dose 50 (LD_{50}) calculated.

Extracts pro-osteogenic bioactivity

High Throughput Assay Procedure

To assess the extracts pro-osteogenic activity a miniaturized high throughput assay was performed using an automated workstation (PerkinElmer, Waltham, USA). Human Mesenchymal Stem Cells (hMSCs) were isolated from the bone marrow of healthy donors at the Galway University Hospital after informed consent and ethical approval. Cells were cultured in growing medium containing α-minimal essential medium (α-MEM, Life technologies), 10% fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin (P/S, Life technologies) supplemented with 1ng ml^{-1} fibroblast growth factor-2 (FGF-2, Peprotech). Culture flasks were maintained at 37°C, 5% CO_2 in humidified atmosphere. Actively proliferating cells were detached from the flasks and resuspended in Basic Medium for osteogenesis (BM) containing phenol red

free Dulbecco's modified eagle medium low glucose (DMEM-LG, Life Technologies), 10% FBS and 1% P/S. Ten thousand cells were seeded in flat bottom 96-well plates (Sarstedt) and incubated overnight at 37°C, 5% CO_2. To evaluate a promotion of differentiation, cells were treated with fungal extracts diluted in Osteogenic Medium (OM) containing BM with 100nmol l^{-1} Dexamethasone, 100µmol l^{-1} Ascorbic acid 2-Phosphate, 10mmol l^{-1} β-glycerophosphate (Sigma-Aldrich, Saint Louis, USA). Crude extracts were tested at four non-toxic concentrations (based on the cytotoxicity screening results), DMSO was kept at 0.5% in experimental wells and controls. Positive control cells were treated with OM+0.5% DMSO and negative control cells with BM+0.5% DMSO. Cells medium was appropriately refreshed 72 h after treatment and early differentiation marker alkaline phosphatase (ALP) measured seven days after treatment. Cells intracellular ALP level was measured using an enzymatic assay involving the reaction enzyme-substrate (*para*-nitrophenilphosphate) as previously described (Bruder *et al.* 1997).

Osteogenic hits re-screening

Fungal extracts that showed to induce cells ALP expression higher than the set threshold (Ctr+ average + 3SD) were selected as positive hits and re-tested for bioactivity. Extracts were tested at 20µg ml^{-1} in two media formulations: added to OM to evaluate promotion of differentiation or in Incomplete Osteogenic Medium (IOM: OM lacking β-glycerophosphate) to evaluate their ability to induce differentiation in absence of one essential component for *in-vitro* hMSCs osteogenic differentiation. Cell seeding and treatment was performed as

241 described before, the ALP level was measured after 7 days differentiation. The
242 cell calcium mineralization was measured as a late differentiation marker after
243 12 days treatment. Due to the longer incubation time, two additional medium
244 changes were performed at days 7 and 10. The calcium mineralized in the
245 extracellular matrix was solubilized in 1mol l^{-1} HCl and quantified as previously
246 described (Jaiswal *et al.* 1997) with a StanBio Calcium Liquicolour Kit (StanBio
247 Calcium assay kit - Thermofisher, Waltham, USA).

248

249 **Statistical analyses**

250 Statistical analyses were performed using PRIMER 7.0 (Plymouth Routines In
251 Multivariate Ecological Research; Clarke and Warwick, 2001). The fungal
252 species biodiversity was evaluated calculating Shannon-Weaver's index (H')
253 and Gini-Simpson's index (1-Lambda). One-way ANOVA and *t*-test on
254 bioactivity results were performed using GraphPad Prism version 7.0
255 (GraphPad Software, La Jolla, California, USA). One-way ANOVA and
256 Bonferroni post-test (significance level of 0.05) was used to compare the data
257 in the osteogenic assessment and OSMAC effect.

258

259 ## Results

260 ***Holothuria poli* associated mycobiota**

261 From 18 samples, obtained by three districts of six animals, 498 fungal strains
262 belonging to 17 taxa were obtained. The retrieved isolates were ascribable to
263 16 genera, all belonging to the phylum Ascomycota (Table 1). *Aspergillus* and

Penicillium were the most represented genera (34% of the total species and 65% of the total microbial load, 25.5% of the total species and 25% of the total microbial load – respectively). Species belonging to *Aspergillus* genus were mainly members of *A. flavipedes* section (*A. micronesiensis, A. poliporycola* and *A. spelaeus* - 18.7% of the *Aspergillus* species and 9.9% of the total microbial load), *A. niger* group (*A. awamori, A. foetidus, A. niger* and *A. tubingensis* - 31.2% of the *Aspergillus* species, 6.6% of the total microbial load) and *A. versicolor* group (*A. creber, A. fructus, A. protuberus, A. sidowii* and *A. versicolor* - 31.2% of the *Aspergillus* species and 6.4% of the total microbial load).

Other genera recorded with more than one species were *Chaetomium* (3 species), *Acremonium,* and *Trichoderma* (2 species). All the animals were colonized by fungi and statistical analyses did not show significant differences amongst animals (p: 0.213, Figure 1).

Please insert here Figure 1

The fungal species occurrence on different animal specimens highlighted two most frequent species, *Aspergillus awamori* and *Penicillium steckii,* isolated from 5 out of 6 animals. Other species detected with high frequency were *Aspergillus insuetus* (4 animals) *A. fructus, A. niger, A. tubingensis, Penicillium brevicompactum, P. citrinum* and *Stachybotrys chartarum* (3 animals).

In regard to the three matrices under analyses (body wall, intestine, faeces), the number of isolated species ranged from 25 associated with the intestine to 22 species isolated from the body wall and faeces. Eighteen of these were isolated from more than one matrix. In terms of global biodiversity, the Shannon

288 index (H') indicated a similar biodiversity level among the 3 districts. Gini-
289 Simpson dominance index pointed out comparable and high species
290 dominance in all the districts (1-Lambda - Table 2).

291 Please insert here Table 2

292 *Aspergillus* was the dominant genus, representing 65% of the total microbial
293 load detected on the three districts and the only ubiquitous genus with 5 species
294 (*A. awamori, A. fructus, A. ochraceus, A. tubingensis* and *A. versicolor*)
295 detected on all districts. These species colonised with a higher rate faeces
296 (average of 304.5±81.9 CFU gdw^{-1}) and intestine content (268.7±95.7 CFU
297 gdw^{-1}), compared to the body wall (65.5±26.8 CFU gdw^{-1}). *Penicillium* was the
298 other district-related dominant genus (25% of the total microbial load): *P. steckii*
299 mainly colonised the faeces (761.7±71.8 CFU gdw^{-1}) and intestine (267.1±42
300 CFU gdw^{-1}), *P. citrinum* colonised the same districts with a higher load in the
301 faeces (118.8±17.2 CFU gdw^{-1}) compared to the intestine (11.9±2 CFU gdw^{-1}),
302 and *P. brevicompactum* occurred with a higher load in the faeces (578.1±81.1
303 CFU gdw^{-1}) than the body wall (15±2.5 CFU gdw^{-1}).

304 Please insert here Table 1

305

306 **Fungal extracts chemical analyses**

307 Sixteen fungal strains were grown for chemical extraction following a
308 preliminary OSMAC approach. Fungi were cultured on two media with sodium
309 chloride, one nutrient rich (SMA) and one nutrient poor (MEClA) and on one
310 nutrient rich medium without sodium chloride (MEA). After four weeks at 24°C,
311 fungal biomass and growing medium metabolites were extracted using both

312 polar and non-polar solvents. Average obtained raw extract dry mass on MEA
313 was 153mg, 335mg on MECIA and 410mg on SMA.

314 The extracts chemical profiling was performed through HPLC: extracts
315 belonging to nine strains generated different metabolic profiles on the three
316 growing media (four examples in Figure 2). Qualitative changes in their
317 metabolic profiles (i.e. peaks at different retention time in the chromatograms
318 of the three media) were reported for all *Penicillium* strains analysed (*P.*
319 *brevicompactum* MUT 1097, *P. chrysogenum* MUT 1115, *P. citrinum* MUT
320 1071, and *P. citrinum* MUT 1105) as well as *Acremonium implicatum* MUT
321 1055, *Acrostalagmus luteoalbus* MUT 1070, *Chaetomium globosum* MUT
322 1013*, Chaetomium* sp. MUT 1035, and *Myrotecium verrucaria* MUT 1069. For
323 these species, SMA medium chromatogram showed the highest number of
324 peaks. Variation in the metabolites production based on the culture conditions
325 was recorded for both different species of the same genus (*Aspergillus insuetus*
326 and *A. protuberus*) and for different strains of the same fungal species (*P.*
327 *citrinum*).

328 Please insert here Figure 2

329

330 **Fungal extracts cytotoxicity**

331 The fungal extracts' cytotoxicity was evaluated by exposing HepG2 cells to
332 seven extracts' concentrations (200ng ml^{-1} - 200µg ml^{-1}) and measuring the
333 viability after 72 h. The established threshold to consider an extract cytotoxic
334 was set at 15% of induced cell death: results showed no extracts' cytotoxicity
335 between 200pg ml^{-1} and 20ng ml^{-1}; two extracts were cytotoxic at 200ng ml^{-1};

336 thee extracts (0.6%) were cytotoxic at 2µg ml^{-1}; 17 (34.4%) at 20µg ml^{-1} and 46
337 extracts (95.6%) were cytotoxic at 200µg ml^{-1}. The LD_{50} recorded (Table 3)
338 ranged from 27.7 to 474.3µg ml^{-1}: 46% of the extracts in the range of 0-100µg
339 ml^{-1}, 27% in the range 100-200µg ml^{-1}, 19% in the range 200-300µg ml^{-1}, 2% in
340 the range 300-400µg ml^{-1} and 6% in the range 400-500µg ml^{-1}. The most
341 cytotoxic extracts were produced by four species of which two belonged to
342 *Chaetomium*, one *Acrostalagmus* and one *Myrotecium* genera (Figure 3).

343 The influence of growing medium on fungal synthesis of cytotoxic compounds
344 (OSMAC effect) was also evaluated. Cell death induced by three extracts
345 belonging to each single species were compared using one-way ANOVA
346 statistical test. All 16 strains demonstrated statistically different cytotoxicity of
347 extracts obtained from the three culture media, for at least one extract
348 concentration tested (Figure S1).

349 Please insert here Figure 3

350 Please insert here Table 3

351

352 **Fungal extracts osteogenesis**

353 In order to evaluate the potential of fungal extracts to promote hMSCs
354 osteogenic differentiation, the 48 extracts were tested using an automated HTS
355 platform. Extracts diluted in DMSO were tested at the four highest nontoxic
356 concentrations defined by the preliminary cytotoxic investigation (less than 15%
357 cell death induced). Possible promoters of osteogenesis were detected from
358 this screening selecting values above a threshold: ALP expression higher than
359 positive control average + three times the standard deviation (Figure S2).

Using this method, 9 extracts were selected as osteogenic promoters and re-screened (MUT 1115-SMA; MUT 1071-SMA; MUT 1097-MEA; MUT 1086-SMA; MUT 1074-SMA; MUT 1091-SMA; MUT 1054-MEA; MUT 1069-MEA; MUT 1035-MECIA). These extracts were manually tested using the same procedure and miniaturized assays for ALP and calcium detection. Extracts were added to IOM to evaluate bioactivity as osteogenic inducers, in absence of β-glycerophosphate in the differentiation medium or added to OM to evaluate improvement of marker expression in presence of all the osteogenic differentiation factors. A significant increase in ALP expression after treatment was detected for four extracts when tested in IOM (Figure 4.A), showing to be able to induce differentiation in absence of β-glycerophosphate in the culture medium. When tested in OM (Figure 4.B), two of these extracts showed to promote a significant increase of ALP compared to the positive control cells treated with OM. In terms of calcium mineralization, no extract was able to induce differentiation when tested in IOM (Figure 4.C) while two extracts showed to improve the cell differentiation by increasing the quantity of mineralized calcium when tested in OM (Figure 4.D). Overall, extracts 1115-SMA and 1071-SMA belonging to *Penicillium chrysogenum* and *P. citrinum*, respectively, showed to improve the osteogenic marker expression ALP and calcium mineralization by hMSCs after treatment.

Please insert here Figure 4

Discussion

Our study demonstrates that the Mediterranean marine echinoderm *Holothuria poli* is widely colonized by fungi in its internal and external body sectors.

384 Isolated fungal strains all belonged to the phylum Ascomycota, confirming it as
385 dominant in the marine environment (Jones *et al.* 2019). Our data show a
386 significantly higher fungal diversity (47 species) compared to that reported from
387 previously investigation on the same animal (Omran and Allam 2013) as well
388 as to that reported from other species of sea cucumbers (Pivkin 2000). Omran
389 and Allam (2013) reported only a single isolate, *Candida albicans*, associated
390 with *H. poli* from the Egyptian Mediterranean Sea, a species not detected in our
391 study. Pivkin (2000) studied sea cucumbers from the Pacific Ocean, reporting
392 25 fungal species from *Eupentacta fraudatrix*, nine from *Apostichopus*
393 *japonicus* and three from *Cucumaria japonica*. The higher diversity that we
394 reported can probably be related to the isolation techniques adopted compared
395 to the previous studies: Pivkin (2000) used agar with holothurian broth, with
396 NaOH added; Omran and Allam (2013) used Sabouraud and blood agar media
397 without the addition of salt. Our isolation strategy implemented the use of CMA,
398 a nutrient rich medium previously reported as optimal for marine fungi isolation
399 (Kossuga *et al.* 2011), added of sea salt to mimic the environmental conditions.
400 In terms of species recurrence, Mediterranean *H.poli* showed four fungal
401 species in common to the Pacific sea cucumber isolated by Pivkin (2000):
402 *Alternaria alternata, A. versicolor, C. sphaerospermum* and *Penicillium*
403 *commune.* In comparison with the mycobiota isolated from other Mediterranean
404 substrates *H. poli* showed more similarities (Fig. 5): ten species in common with
405 the brown algae *Padina pavonica* (Garzoli *et al.* 2018), nine in common with
406 the green algae *Flabellia petiolata* (Gnavi *et al.* 2017), nine with the seagrass
407 *Posidonia oceanica* (Panno *et al.* 2013), eight with the sponge *Psammocinia*
408 sp. (Paz *et al.* 2010) and five with decaying woods (Garzoli *et al.* 2015). Most

409 common isolated fungal species from Mediterranean marine substrates are *T.*
410 *harzianum* and *P. brevicompactum*, respectively isolated in the past from
411 Oceanic sponges and ascidians (Yamada *et al.* 2014; Vacondio *et al.* 2015)
412 and from Oceanic algae and sponges (Alves 2019; Bovio *et al.* 2019). Such
413 common species are likely to be highly adapted to the marine environment
414 showing a high potential in substrate colonization, therefore their specific role
415 in association with animals, plants and algae should be thoroughly investigated
416 to identify possible key marine species.

417 Please insert here Figure 5

418 Eleven species isolated in this study represent new records for the marine
419 environment worldwide: *A. creber, A. foetidus, A. fructus, A. micronesiensis, A.*
420 *spelaeus, Auxarthron ostraviense, Chaetomium subaffine, Emericella*
421 *quadrilineata, Myriodontium. keratinophilum, P. adametzii,* and *Trichoderma*
422 *epimyces*. Several of them have been previously described as soil inhabitants
423 (Pitt 1979; Doveri *et al.* 2013; López-Quintero *et al.* 2013; Nováková *et al.* 2014)
424 or present in house dust (Samson *et al.* 2014a). *Auxarthron ostraviense, M.*
425 *keratinophilum* and *E. quadrilineata* are usually reported from soils and are
426 described as weak human pathogens (Maran *et al.* 1985; Gugnani *et al.* 2007;
427 Hubka *et al.* 2013). Further records on new substrates are needed to elucidate
428 their presence in the marine environment.

429 The isolated fungal community was characterized by the dominance of
430 *Penicillium* and *Aspergillus* genera, as previously detected on other
431 Mediterranean substrates (Paz *et al.* 2010; Panno *et al.* 2013). Species
432 belonging to these genera are highly sporulating and able to adapt to extremely

433 disparate environmental conditions such as Antarctica, Atacama desert or deep
434 seas (Godinho *et al.* 2015; Nagano *et al.* 2017; Santiago *et al.* 2018), and
435 therefore are likely to survive in shallow waters and colonize sessile
436 echinoderms. The presence of recurrent species such as *A. awamori* and *P.*
437 *steckii* suggests a higher adaptation of these taxa to live associated to an
438 animal producing antifungal molecules and a possible selection of the
439 mycobiota operated by the animal.

440 Our study confirms the pharmaceutical bioactivity of small molecule extracts
441 obtained by fungal strains associated to *H. poli.* Cytotoxicity assessment
442 against hepatocarcinoma cells (HepG2) showed the ability of several strains to
443 produce highly cytotoxic metabolites, a concentration-related bioactivity and the
444 medium influence in the biosynthesis of these compounds. Producers of the
445 most cytotoxic extracts were members of the *Chaetomium* genus, previously
446 reported to synthetize several azaphilones, molecules produced by
447 *Chaetomium globosum* with selective cytotoxicity against leukemia HL60,
448 leukemia L1210 and KB epidermoid carcinoma cell lines (Yamada *et al.* 2012).
449 The marine fungi ability to synthetize human cells' cytotoxic compounds clearly
450 show their potential for the treatment of cancer-based diseases. Future
451 analyses on cytotoxic extracts will involve the investigation of bioactivity
452 retention against other cancer cell lines and the extracts' chemical investigation
453 to purify the molecules responsible of this bioactivity.

454 Regarding the fungal extracts' bioactivity toward human Mesenchymal Stem
455 Cells, our initial high throughput screening showed nine extracts able to induce
456 differentiation of hMSCs into bone cell progenitors. The positive hits validation
457 was performed by re-testing the extracts and evaluating their specific bioactivity

458 as inducers or promoters of osteogenic differentiation. This was done by testing
459 the extracts diluted in complete osteogenic medium containing the full cocktail
460 of pro-osteogenic molecules for *in-vitro* differentiation or in incomplete
461 osteogenic medium lacking β-glycerophosphate. Cells differentiation after
462 treatment was evaluated by measuring two osteogenic markers: intracellular
463 alkaline phosphatase production and cells calcium mineralization. Extracts
464 obtained by *P. chrysogenum* (MUT 1115) and *P. citrinum* (MUT 1071) induced
465 and promoted osteogenic differentiation as showed by increased level of
466 intracellular ALP and increased amount of calcium mineralized in the
467 extracellular matrix, process leading to bone formation, demonstrating great
468 potential for the discovery of new drug candidates for regenerative medicine.
469 While this is the first record for *P. chrysogenum* producing compounds with pro-
470 osteogenic bioactivity, *P. citrinum* was previously reported to synthetize
471 mevastatin (Endo *et al.* 1976), a molecule belonging to a class of metabolites,
472 statins, known for their bioactivity as cholesterol-lowering and documented to
473 stimulate the formation of new bone tissue (Mundy *et al.* 1999; Morse *et al.*
474 2018).

475 The culture conditions' influence on fungal metabolite production (OSMAC
476 effect) was demonstrated both with chemical profiling and detected extracts
477 bioactivity. The variable cytotoxic bioactivity of extracts belonging to the same
478 strain growing on different media was confirmed by the extracts chemical
479 analyses that showed chromatograms variability. Most effective medium for the
480 biosynthesis of bioactive compounds was SMA, responsible for the production
481 of the most cytotoxic extracts in terms of number and induction of cell death,
482 and the main medium where osteogenic metabolites were synthesized. Once

again is demonstrated that the discovery of new fungal secondary metabolites for biotechnological applications is strictly linked to the modification of culture conditions to trigger different metabolic pathways and induce the production of the widest fungal metabolome.

In this study we prove as demonstrated before (Lagarde *et al.* 2018) the fungal intraspecific variation in the production of bioactive compounds: different strains of *P. citrinum* isolated in this study showed different chromatographic profiles and different detected bioactivity as cytotoxicity and pro-osteogenic compounds. This evidence highlights the importance of strains evaluation for the discovery of new compounds for specific pharmaceutical applications in order to widen the chances to discover strains with pharmaceutical potential.

To the best of our knowledge, this is the first record on marine fungal extracts inducing hMSCs osteogenic differentiation in comparison to the established inducer, β-glycerophosphate, and potential producers of new molecules for regenerative medicine. Positive extracts will therefore be further analysed to identify the responsible molecules associated with the detected bioactivity and describe potential drug candidates. Further evidences of cytotoxic selectivity and hMSC osteogenic induction by the metabolites produced by our strains are needed to evaluate progression towards their use in targeted cell therapies, particularly in diseases such as cancer (Cragg *et al.* 2009), osteoporosis and osteogenesis imperfecta (Grunevald *et al.* 2014; Besio and Forlino 2015, Gagliardi *et al.* 2017). Furthermore, the optimized High Throughput assays developed in the present study allowed us to test our extracts library in a time and cost effective way and are a valuable tool to foster studies of cell-related bioactivity from wide natural compounds libraries.

To conclude, a peculiar fungal community with an interesting metabolic potential was recorded on *Holothuria poli*, increasing the knowledge about animal fungal association in the Mediterranean Sea, a marine biodiversity hotspot whose microbial community is substantially underestimated. The importance of culture based fungal community investigation in biodiscovery research was once again underlined: the development of future studies to unlock fungal biotechnological potential is strictly connected to the conservation of pure strains in culture.

Acknowledgments:

We acknowledge grant support from CRT Foundation, Science Foundation Ireland - Strategic Research Cluster 09/SRC/B1794 and Irish Research Council Postgraduate Scholarship GOIPG/2016/1113. The authors acknowledge the facilities and scientific and technical assistance of the Genomics and Screening Core at the National University of Ireland Galway, a facility that is funded by NUIG and the Irish Government's Programme for Research in Third Level Institutions, Cycles 4 and 5, National Development Plan 2007-2013.

Conflict of Interest:

The authors have no conflict of interest to declare

References

530 Alves, A. (2019) Biodiversity of *Penicillium* species from marine environments
531 in Portugal and description of *Penicillium lusitanum* sp. nov., a novel
532 species isolated from sea water. *Int J Syst Evol Microbiol* **39**, 3014-3021.

533 Alves, H., Dechering, K., Van Blitterswijk, C., and De Boer, J. (2011) High-
534 throughput assay for the identification of compounds regulating
535 osteogenic differentiation of human mesenchymal stromal cells. *PLoS*
536 *ONE* **6**, e26678.

537 Besio, R., and Forlino, A. (2015) Treatment options for osteogenesis
538 imperfecta. *Exper. Opin. Orphan Drugs* **3**, 165-181.

539 Besio, R., Chow, C. W., Tonelli, F., Marini, J. C., and Forlino, A. (2019a) Bone
540 biology: insights from osteogenesis imperfecta and related rare fragility
541 syndromes. *The FEBS J* doi: 10.1111/febs.14963.

542 Besio, R., Garibaldi, N., Leoni, L., Cipolla, L., Sabbioneda, S., Biggiogera, M.,
543 Mottes, M., Aglan, M., Otaify, G.A., Temtamy, S.A., Rossi, A., and Forlino
544 A. (2019b) Cellular stress due to impairment of collagen prolyl
545 hydroxylation complex is rescued by the chaperone 4-phenylbutyrate. *Dis*
546 *models mech.* **12**, doi:10.1242/dmm.038521.

547 Bovio, E., Garzoli, L., Poli, A., Luganini, A., Villa, P., Musumeci, R.,
548 McCormack, G.P., Cocuzza, C.E., Gribaudo, G., Mehiri, M., and Varese
549 G.C. (2019) Marine fungi from the sponge G*rantia compressa*:
550 biodiversity, chemodiversity, and biotechnological potential.*Mar drugs* **17**
551 doi: 10.3390/md17040220.

552 Bode, H.B., Bethe, B., Höfs, R., and Zeeck, A. (2002) Big effects from small
553 changes: possible ways to explore nature's chemical diversity.
554 *ChemBioChem* **3**, 619-627.

555 Bruder, S.P., Jaiswal, N., and Haynesworth, S.E. (1997) Growth kinetics, self-
556 renewal, and the osteogenic potential of purified human mesenchymal
557 stem cells during extensive subcultivation and following cryopreservation.
558 *J Chem Biochem* **64**, 278-294.

559 Clarke, K.R., and Warwick, R.M. (2014) Change in marine communities: an
560 approach to statistical analysis and interpretation, second ed. PRIMER-E
561 Ltd., Plymouth.

562 Cragg, G.M., Grothau, P.G., and Newman, D.J. (2009) Impact of Natural
563 Products on Developing New Anti-Cancer Agents. *Chem Rev* **109**, 3012-
564 3043.

565 Doveri, F., Sarrocco, S., and Vannacci, G. (2013) Studies on three rare
566 coprophilous plectomycetes from Italy. *Mycotaxon* **124**, 279-300.

567 Ebel, R. (2012) 20 natural products from marine derived fungi, In Marine
568 Fungi and Fungal-like Organisms eds. Jones, E.B.G., and Pang, K.L. pp
569 411. Berlin: Walter de Gruyter.

570 Endo, A., Masao, K., and Yoshio, T. (1976) ML-236A, ML-236B, and ML-
571 236C, new inhibitors of cholesterogenesis produced by *Penicillium*
572 *citrinum*. *J. Antibiot* **29**, 1346-1348.

573 Gagliardi, A., Besio, R., Carnemolla, C., Landi, C., Armini, A., Aglan, M.,
574 Otaify, G., Temtamy S.A., Forlino, A., Bini, L., and Bianchi, L. (2017)

575 Cytoskeleton and nuclear lamina affection in recessive osteogenesis
576 imperfecta: A functional proteomics perspective. *J proteomics* **167**, 46-59.

577 Garzoli, L., Gnavi, G., Tamma, F., Tosi, S., Varese, G.C., and Picco, A.M.,
578 (2015) Sink or swim: updated knowledge on marine fungi associated with
579 wood substrates in the Mediterranean Sea and hints about their potential
580 to remediate hydrocarbons. *Prog Oceanogr* **137**, 140-148.

581 Garzoli, L., Gnavi, G., Varese, G.C., and Picco, A.M. (2014) Mycobiota
582 associated with the rhodophyte alien species *Asparagopsis taxiformis*
583 (Delile) Trevisan de Saint-Léon in the Mediterranean Sea. *Mar Ecol* **36**,
584 959-968.

585 Garzoli, L., Poli, A., Prigione, V., Gnavi, G., and Varese, G.C. (2018)
586 Peacock's tail with a fungal cocktail: first assessment of the mycobiota
587 associated with the brown alga *Padina pavonica*. *Fungal Ecol* **35**, 87-97.

588 Gnavi, G., Garzoli, L., Poli, A., Burgaud, G., Prigione, V., and Varese, G.C.
589 (2017) The culturable mycobiota of *Flabellia petiolata*: First survey of
590 marine fungi associated to a Mediterranean green alga. *Plos One* **12**,
591 doi.org/10.1371/journal.pone.0175941.

592 Godinho, V.M., Gonçalves, V.N., Santiago, I.F., Figueredo, H.M., Vitoreli,
593 G.A., Schaefer, C.E.G.R., Barbosa, E.C., Oliveira, J.G., Alves, T.M.A.,
594 Zani, C.L., Wedge, D.E., Duke, S.O., Ali, A., Rosa, C.A., and Rosa, L.H.
595 (2015) Diversity and bioprospection of fungal community present in
596 oligotrophic soil of continental Antarctica. *Extremophiles* **111**, 585-596.

597 Gruenwald, K., Castagnola, P., Besio, R., Dimori, M., Chen, Y., Akel, N. S.,
598 Swain, F.L., Skinner, R.A., Eyre D.R., Gaddy, D., Suva, L.J., and Morello

599 R. (2014) Sc65 is a novel endoplasmic reticulum protein that regulates
600 bone mass homeostasis. *J Bone Miner Res* **29**, 666-675.

601 Gugnani, H.C., Paliwa-Joshi, A., Rahman, H., Padhye, A.A., Singh, T.S.K.,
602 Das, T.K., Khanal, B., Bajaj, R., Rao, S., and Chukhani R. (2007)
603 Occurrence of pathogenic fungi in soil of burrows of rats and of other sites
604 in bamboo plantations in India and Nepal. *Mycoses* **50**, 507-511.

605 Hubka, V., Dobiasova, S., Lyskova, P., Mallatova, N., Chlebkova, J.,
606 Skorepova, M., and Kolarik, M. (2013) *Auxarthron ostraviense* sp. nov.,
607 and *A. umbrinum* associated with non-dermatophytic onychomycosis.
608 *Med Mycol* **51**, 614-624.

609 Imhoff, J.F. (2016) Natural products from marine fungi – still an
610 underrepresented resource. *Mar Drugs* **14**, 19.

611 Ismail, H., Lemriss, S., Ben Aoun, Z., Mhadhebi, L., Dellai, A., Kacem, Y.,
612 Boiron, P., and Bouraoui, A. (2008) Antifungal activity of aqueous and
613 methanolic extracts from the Mediterranean sea cucumber, *Holothuria*
614 *polii*. *J Mycol Med* **18**, 23-26.

615 Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S.P. (1997)
616 Osteogenic differentiation of purified, culture-expanded human
617 mesenchymal stem cells in vitro. *J Cell Biochem* **64**, 295-312.

618 Jones, E.B.G. (2011) Fifty years of marine mycology. *Fungal Divers* **50**, 73-
619 112.

620 Jones, E.B.G., and Pang, K.L. (2012) Marine Fungi and Fungal-like
621 Organisms. Berlin: Walter de Gruyter.

622 Jones, E.B.G., Pang, K.L., Abdel-Wahab, M.A., Scholz, B., Hyde, K.D.,
623 Boekhout, T., Ebel, R., Rateb, M.E., Henderson, L., Sakayaroj, J.,
624 Suetrong, S., Dayarathne, M.C., Kumar, V., Raghukumar, S., Sridhar,
625 K.R., Bahkali, A.H., Gleason, F.H. (2019) An online resource for marine
626 fungi. *Fungal Divers* **96**, 347-433.

627 Kossuga, M.H., Romminger, S., Xavier, C., Milanetto, M.C., doValle, M.Z.,
628 Pimenta, E.F., Morais, R.P., de Carvalho, E., *et al.* (2011) Evaluating
629 methods for the isolation of marine-derived fungal strains and production
630 of bioactive secondary metabolites. *Rev bras Farmacogn* **22**, 257-267.

631 Lagarde, A., Millot, M., Pinon, A., Liagre, B., Girardot, M., Imbert, C., Ouk,
632 T.S., Jargeat, P., and Mambu, L. (2018) Antiproliferative and antibiofilm
633 potentials of endolichenic fungi associated with the lichen *Nephroma*
634 *laevigatum*. J. Appl. Microbiol. 126, 1044-1058

635 López-Quintero, C.A., Atanasova, L., Franco-Molano, A.E., Gam, W., Komon-
636 Zelazowska, M., Theelen, B., and Druzhinina, I. (2013) DNA barcoding
637 survey of *Trichoderma* diversity in soil and litter of the Colombian lowland
638 Amazonian rainforest reveals *Trichoderma strigosellum* sp. nov. and other
639 species. *Anton Leeuw Int J G* **104**, 657-674.

640 Maran, A.G., Kwong, K., Milne, L.J., and Lamb, D. (1985) Frontal sinusitis
641 caused by *Myriodontium keratinophilum*. *Brit Med J* **290**, 207.

642 Morse, L. R., Coker, J., and Battaglino, R. A. (2018) Statins and bone health:
643 A mini review. *Actual osteol* **14**, 31-35.

644 Mundy, G., Garrett, R., Harris, S., Chan, J., Chen, D., Rossini, G., and
645 Gutierrez, G. (1999) Stimulation of bone formation in vitro and in rodents
646 by statins. *Science* **286**, 1946-1949.

647 Nagano, Y., Miura, T., Nishi, S., Lima, A.O., Nakayama, C., Pellizari, V.H.,
648 and Fujikura, K. (2017) Deep-Sea Research Part II Fungal diversity in
649 deep-sea sediments associated with asphalt seeps at the Sao Paulo
650 Plateau. *Deep Res Part II* **146**, 59-67.

651 Nováková, A., Hubka, V., and Dudová, Z. (2014) New species in *Aspergillus*
652 section Fumigati from reclamation sites in Wyoming (U.S.A.) and revision
653 of A*. viridinutans* complex. *Fungal Divers* **64**, 253-274.

654 Omran, N.E.E., and Allam, N.G. (2013) Screening of microbial contamination
655 and antimicrobial activity of sea cucumber *Holothuria polii*. *Toxicol Ind*
656 *Health* **29**, 944–954.

657 Panno, L., Bruno, M., Voyron, S., Anastasi, A., Gnavi, G., Miserere, L., and
658 Varese, G.C. (2013) Diversity, ecological role and potential
659 biotechnological applications of marine fungi associated to the seagrass
660 *Posidonia oceanica*. *N Biotechnol* **30**, 686-694.

661 Paz, Z., Komon-Zelazowska, M., Druzhinina, I.S., Aveskamp, M.M.,
662 Shnaiderman, A., Aluma, Y., Carmeli, S., Mitchnik I., and Yarden, O.
663 (2010) Diversity and potential antifungal properties of fungi associated
664 with a Mediterranean sponge. *Fungal Divers* **42**, 17-26.

665 Pitt, J.I. (1979) The genus Penicillium and its teleomorphic states
666 *Eupenicillium* and *Talaromyces*. London: Academic Press Inc. Ltd., pp. 1-
667 148.

668 Pivkin, M.V. (2000) Filamentous fungi associated with holothurians from the
669 sea of Japan, off the Primorye coast of Russia. *Biol Bull* **198**, 101-109.

670 Prince, L., and Samuel, P. (2015) A study on the diversity of marine fungi
671 along the south east coast of Tamilnadu – A statistical analysis. *Int J Curr*
672 *Microbiol App Sci* **4**, 559-574.

673 Samson, R., Visagie, C., and Houbraken, J. (2014) Species diversity in
674 *Aspergillus*, *Penicillium* and *Talaromyces*. *Stud Mycol* **78**.

675 Samson, R., Visagie, C., Houbraken, J., Hong, S., Hubka, V., Klaassen, C.,
676 and Varga, J. (2014). Phylogeny, identification and nomenclature of the
677 genus *Aspergillus*. *Stud Mycol* **78**, 141-173.

678 Santiago, I.F., Goncalves, V.N., Gomez-Silva, B., Galetovic, A., Go, B., and
679 Rosa, L.H. (2018) Fungal diversity in the Atacama Desert *Anton Leeuw*
680 *Int J G* **111**, 1345-1360.

681 Schofield, M. M., Jain, S., Porat, D., Dick, G. J., and Sherman, D. H. (2015)
682 Identification and analysis of the bacterial endosymbiont specialized for
683 production of the chemotherapeutic natural product ET-743. *Environ*
684 *microbiol* **17**, 3964-3975.

685 Seifert, K.A., Morgan-Jones, G., Gams, W., and Kendrick, B. (2011) The
686 genera of Hyphomycetes-2011 update. *Persoonia* **27**, 119-129.

687 Silber, J., Kramer, A., Labes, A., and Tasdemir, D. (2016) From discovery to
688 production: biotechnology of marine fungi for the production of new
689 antibiotics. *Mar Drugs* **14**, 137.

690 Syed, B.A., (2019) Comparative studies on the production of statins using
691 different microbial strains. BioTechnologia **100**, 251-262.

692 Vacondio, B., Birolli, W.G., Ferreira, I.M., Seleghim, M.H.R., Gonçalves, S.,
693 Vasconcellos, S.P., and Porto, A.L.M. (2015) Biocatalysis and Agricultural
694 Biotechnology Biodegradation of pentachlorophenol by marine-derived
695 fungus *Trichoderma harzianum* CBMAI 1677 isolated from ascidian
696 *Didemnun ligulum*. *Biocatal Agric Biotechnol* **4**, 266-275.

697 Yamada, T., Jinno, M., Kikuchi, T., Kajimoto, T., Numata, A., andTanaka, R.,
698 (2012) Three new azaphilones produced by a marine fish-derived
699 *Chaetomium globosum*. *J Antibiot* **65**, 413-417.

700 Yamada, T., Mizutani, Y., Umebayashi, Y., Inno, N., Kawashima, M., Kikuchi,
701 T., and Tanaka, R. (2014) Tandyukisin , a novel ketoaldehyde decalin
702 derivative , produced by a marine sponge-derived *Trichoderma*
703 *harzianum*. *Tetrahedron Lett* **55**, 662-664.

704 WoRMS (2020). *Holothuria (Roweothuria) poli* Delle Chiaje, 1824. Accessed
705 at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=124525 on
706 2020-01-16

707

Table 1: Marine fungal species recovered from *Holothuria polii* in the Tunisian Sea

Taxa	MUT code	Animal*						Matrix*		
		1	2	3	4	5	6	B	I	S
Acremonium alternatum Link 1809	1054						17.02		17.02	
Acremonium implicatum (J.C. Gilman & E.V. Abbott) W. Gams 1975	1055				12.82			12.82		
Acrostalagmus luteoalbus Pethybr. 1919	1070 2130		14.95	21.22				14.95	21.22	
Alternaria alternata (Fr.) Keissl. 1912	856			63.66					63.66	
Aspergillus awamori Nakaz. 1907	2012 2060 2099 2191	86.29	384.6	487.8		163.46	17.02	86.29	483.90	569.08
Aspergillus creber Jurjevic, S.W. Peterson & B.W. Horn 2012	1999 2026		53.41					14.95		38.46
Aspergillus foetidus Thom & Raper 1945	2035					100.59				100.59
Aspergillus fructus Jurjevic, S.W. Peterson & B.W. Horn 2012	2175 2187 2200 2203	44.86	130.34	466.88				32.21	466.88	142.92
Aspergillus insuetus (Bainier) Thom & Church 1929	1085 2054 2092	15.47	29.91	21.22		24.74		42.16	49.18	
Aspergillus micronesiensis Visagie, Hirooka & Samson 2014	1990 1992	34.51	59.82					94.34		
Aspergillus nidulans (Eidam) G. Winter 1884	2165			21.22					21.22	
Aspergillus niger Tiegh. 1867	2082		1692.3	41.99			87.24			1821.5
Aspergillus ochraceus G. Wilh. 1877	2036 2086 2096 2105		680.06	122.79				161.4	295.3	346.15
Aspergillus polyporicola Hubka, A. Nováková, M. Kolarík & S.W. Peterson 2015	1456					528.12				528.12
Aspergillus protuberus Munt.-Cvetk. 1968	1091					24.97			24.97	
Aspergillus pseudodeflectus Samson & Mouch. 1975	2040		38.46							38.46
Aspergillus sp.	2106			21.22		12.48			33.70	
Aspergillus spelaeus A. Nováková, Hubka, M. Kolarík & S.W. Peterson 2015	1457 1993					352.08	17.02		17.02	352.08
Aspergillus sydowii (Bainier & Sartory) Thom & Church 1926	2072 2102		14.95			12.48		14.95	12.48	
Aspergillus tubingensis Mosseray 1934	1074 2043 2047	41.41	76.92				67.06	33.01	34.04	118.33
Aspergillus versicolor (Vuill.) Tirab. 1908	1086 2052 2190		361.11	63.66				14.95	63.66	346.15

709

Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud 1918	2186					12.48			12.48	
Auxarthron ostraviense Hubka, Dobiášová & M. Kolařík 2012	1556		14.95					14.95		
Cadophora luteo-olivacea (J.F.H. Beyma) T.C. Harr. & McNew 2003	1073			31.49						31.49
Chaetomium globosum Kunze 1817	1013		14.95					14.95		
Chaetomium sp.	1035			21.22					21.22	
Chaetomium subaffine Sergeeva 1961	1015			21.22			17.02		38.24	
Cladosporium sphaerospermum Penz. 1882	1454	27.60								27.6
Emericella quadrilineata (Thom & Raper) C.R. Benj. 1955	2019 2025		271.28	21.22				44.87	247.63	
Myrothecium verrucaria (Alb. & Schwein.) Ditmar 1813	1069		14.95					14.95		
Myriodontium keratinophilum Samson & Polon. 1978	1443				12.82	12.48		12.82	12.48	
Paecilomyces lilacinus (Thom) Samson 1974	992		29.91					29.91		
Penicillium adametzii K.M. Zaleski 1927	2107		38.46							38.46
Penicillium antarcticum A.D. Hocking & C.F. McRae 1999	2125 2170	41.41					17.02		17.02	41.41
Penicillium brevicompactum Dierckx 1901	1097 2061 2097 2140		514.95		27.83	50.29		14.95		578.13
Penicillium chrysogenum Thom 1910	1115		29.91					29.91		
Penicillium citreonigrum Dierckx 1901	2062		269.23							269.23
Penicillium citrinum Thom 1910	1071 1105	13.80	11.91	104.99					11.91	118.79
Penicillium commune Thom 1910	2120	15.47							15.47	
Penicillium corylophilum Dierckx 1901	2173	69.01								69.01
Penicillium oxalicum Currie & Thom 1915	2094 2149		44.87	42.44				44.87	42.44	
Penicillium roseopurpureum Dierckx 1901	2146	86.29						86.29		
Penicillium steckii K.M. Zaleski 1927	1453 2109		423.07	317.65	13.91	12.48	261.73		267.14	761.72
Pleosporales sp.	2081		38.46							38.46
Stachybotrys chartarum (Ehrenb.) S. Hughes 1958	1554 1555	13.80	38.46		12.82			12.82		52.26
Trichoderma epimyces Jaklitsch 2008	2022					12.48			12.48	
Trichoderma harzianum Rifai 1969	2009		192.3							192.3
Mean animal fungal load (CFU/gdw)		40.83	203.1	111.3	16.05	101.5	62.65	38.34	92.12	287.9
Number of detected species		12	27	17	5	13	8	22	25	22

*species detected in each animal (1 to 6) and matrix (B= Body wall; I= Intestine; S= Faeces)

710
711

Table 2: Biodiversity within districts: Shannon-Weaver's index (H') and Gini-Simpson's index (1-Lambda)

Matrix	N° Species	H'	1-Lambda
B	22	3.007	0.954
I	25	3.071	0.946
S	22	3.013	0.944

B=Body wall; I=Intestine; S=Faeces

712
713
714
715

Table 3 Cytotoxicity of crude extracts on human hepatocarcinoma cell line (HepG2)

		Growing medium		
Species	**MUT code**	**SMA**	**MECIA**	**MEA**
Acremonium alternatum	1054	>200	>100	>200
Acremonium implicatum	1055	>200	>200	>200
Aspergillus insuetus	1085	>200	>100	62.1
Aspergillus protuberus	1091	93.6	>100	71.3
Aspergillus tubingensis	1074	>100	>100	>100
Aspergillus versicolor	1086	82.7	>400	70.4
Cadophora luteo-olivacea	1073	>100	>300	>400
Chaetomium globosum	1013	37.4	60.0	56.3
Chaetomium sp.	1035	49.7	>100	49.4
Chaetomium subaffine	1015	>100	>200	>200
Myrotecium verrucaria	1069	54.5	39.5	27.7
Nectria inventa	1070	41.9	74.3	53.0
Penicillium brevicompactum	1097	80.4	>100	55.0
Penicillium chrysogenum	1115	>100	60.9	70.8
Penicillium citrinum	1071	>100	>400	91.4
Penicillium citrinum	1105	>100	>200	82.7

Cells were treated for 72 hours with increasing concentration of crude extract (from 0.2ng ml^{-1} to 0.2mg ml^{-1}) or controls. Values are calculated as LD_{50} in µg ml^{-1}

716
717
718
719
720
721
722
723
724
725
726
727
728
729
730

Figure Captions

Figure 1. PCA analyses of animals and districts. Triangle pointing upward is Body wall (B); triangle pointing downward is Intestine (I); square is Sand (S).

Figure 2. Fungal extracts HPLC chromatographic profiles at 280nm. Crude extracts obtained by fungal growth on different media (Soy Mannitol Agar, SMA; Malt Extract Chloride Agar, MECIA; Malt extract Agar, MEA) were analyzed by HPLC to detect variability in the metabolites content. In the figure are showed the extracts profiles on the different growing media of two strains belonging to the same genera (*Aspergillus insuetus, Aspergillus protuberus*) and two different strains of the same species (*Penicillium citrinum*).

Figure 3. Fungal extracts cytotoxicity toward hepatocarcinoma (HepG2) cells. The extracts' cytotoxicity of four of 16 fungal strains, encompassing the most cytotoxic, are here showed. Five thousand cells were seeded in 96-well plates and treated with fungal extracts for 72 hours at 37℃, 5% CO_2. Extracts dissolved in DMSO were diluted in cell growing medium to reach seven increasing concentrations to treat the cells. Positive control cells (ctr+, dashed line) were treated with growing medium +0.5% DMSO while negative control cells (ctr-, dotted line) were treated with growing medium +10% DMSO. Cell viability after treatment was measured by adding 10% v/v of resazurin based dye (alamarBlue) and incubating at 37℃, 5% CO_2 for 6hours. Fluorescence was then read at 531nm excitation wavelength, 572nm emission wavelength. Light grey line with round dot is extract from MEA medium; dark grey line with triangle dot is extract from MECLA medium; black line with rhomboidal dot is extract from SMA medium.

Figure 4. Positive hit extracts re-tested for osteogenic bioactivity. hMSCs differentiation after fungal extracts treatment was evaluated by measuring two markers. Early differentiation marker alkaline phosphatase (ALP) expression was measured 7 days after treatment. Cells were exposed to 20µg ml^{-1} fungal extract dissolved in Incomplete Osteogenic medium to evaluate their ability to induce differentiation in absence of β-glycerophosphate in the medium (A) or in Osteogenic Medium to evaluate an improvement on the differentiation induced by the standard differentiation medium (B). Late differentiation marker, matrix mineralized calcium, was measured 12 days after treatment. Cells were treated with extracts dissolved in IOM (C) or OM (D). The experiment was carried out on experimental triplicates. Results are presented as the mean ± SD of 3 technical replicates, * = $p \leq 0.05$ calculated using ANOVA one-way with Bonferroni post-test.

Figure 5. Fungal species occurrence on Mediterranean substrates. The Venn diagram highlights common fungal species between *Holothuria poli* and other Mediterranean substrates.

Figures

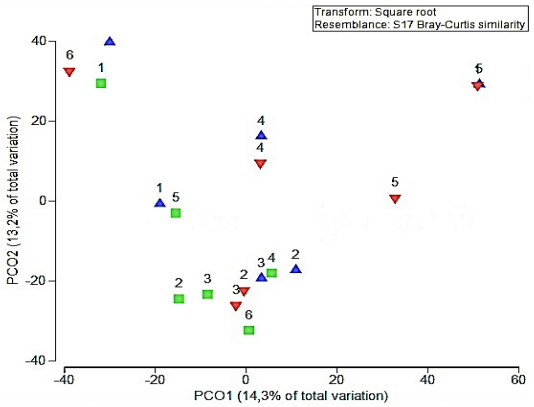


Figure 1

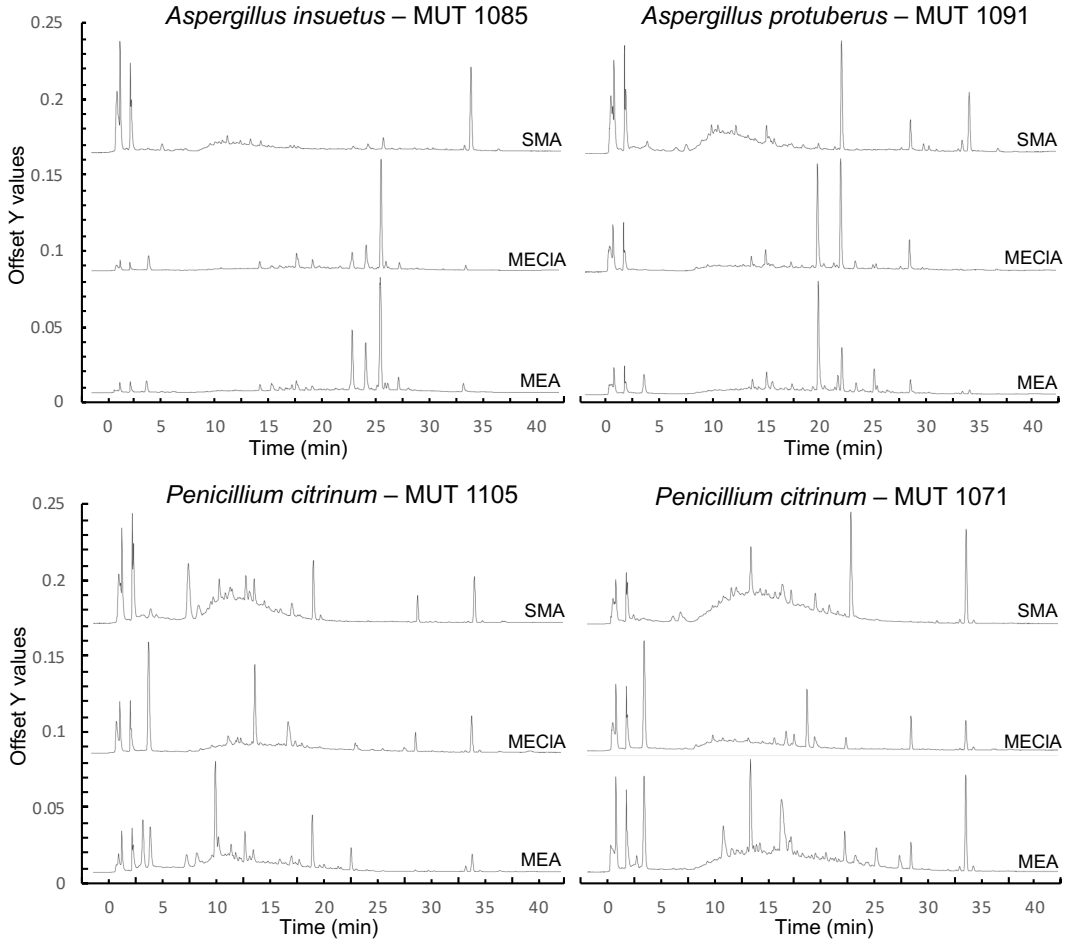
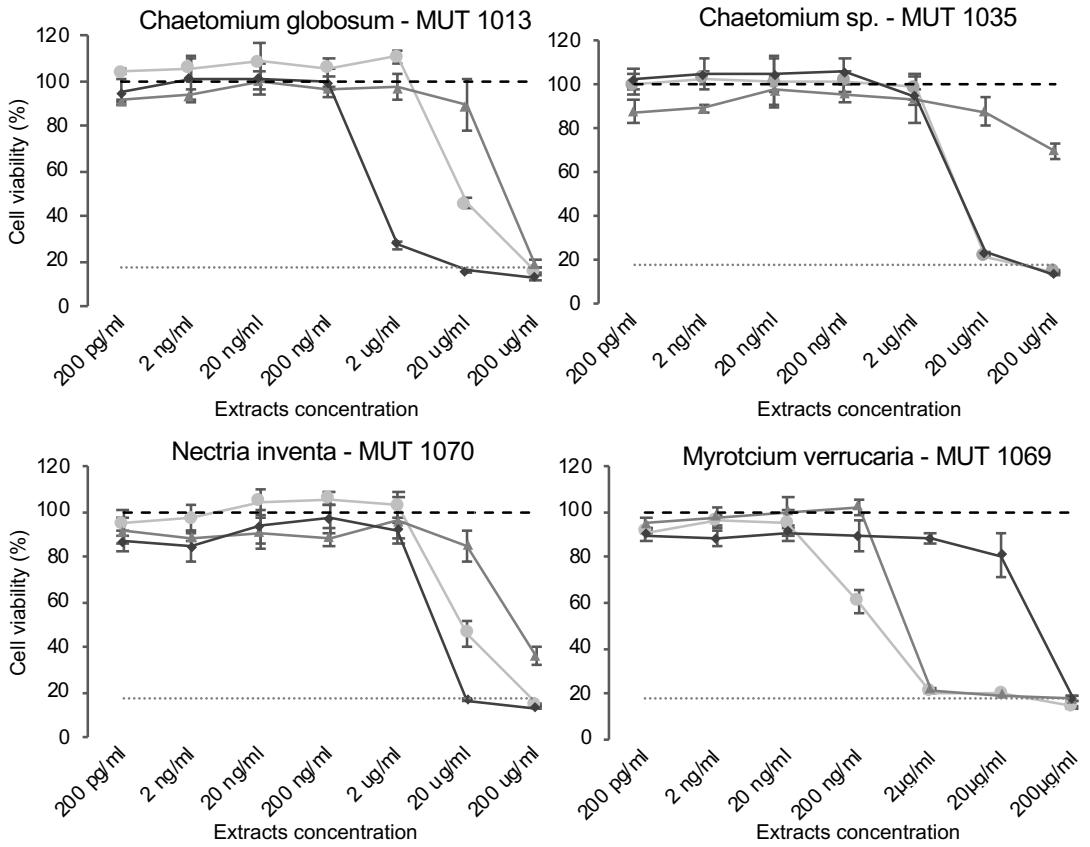
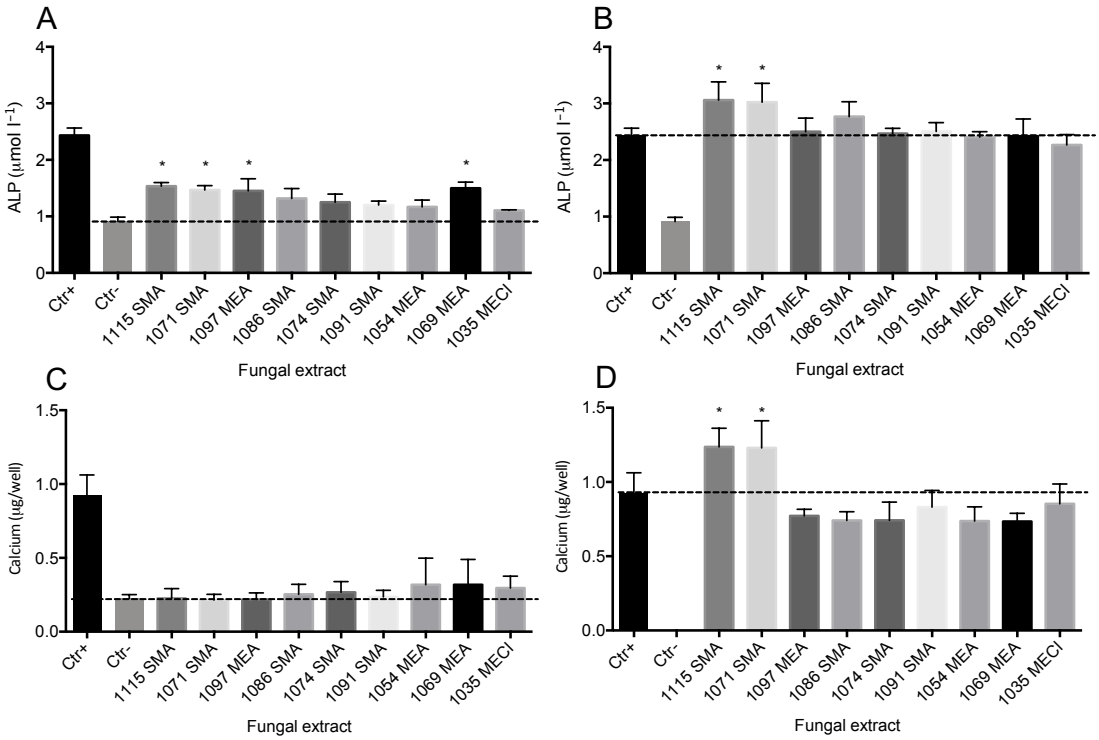


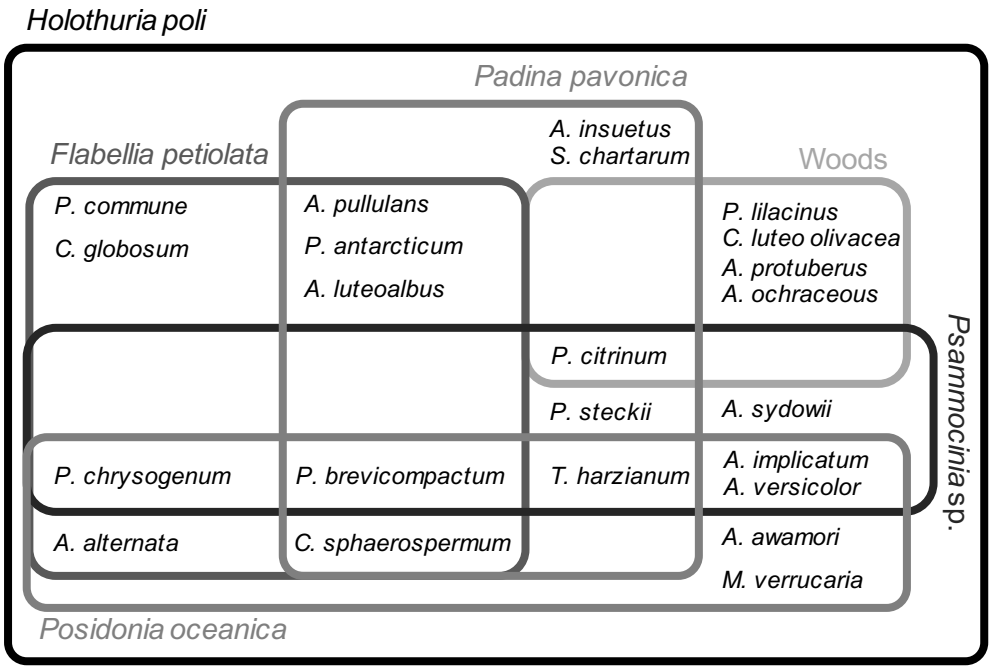
Figure 2



785 Figure 3
786



787 Figure 4
788
789



790
791 Figure 5
792
793