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The culturable mycobiota associated with the Mediterranean sponges *Aplysina cavernicola, Crambe crambe* and *Phorbas tenacior*

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Abstract

Marine fungi are part of the huge and understudied biodiversity hosted in the sea. To broaden the knowledge on fungi inhabiting the Mediterranean Sea and their role in sponge holobiont, three sponges namely *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior* were collected in Villefranche sur Mer, (France) at about 25 m depth. The fungal communities associated with the sponges were isolated using different techniques to increase the numbers of fungi isolated. All fungi were identified to species level giving rise to 19, 13 and 3 species for *P. tenacior*, *A. cavernicola* and *C. crambe*, respectively. Of note, 35.7% and 50.0% of the species detected were either reported for the first time in the marine environment or in association with sponges. The mini-satellite analysis confirmed the uniqueness of the mycobiota of each sponge, leading to think that the sponge, with its metabolome, may shape the microbial community.

Keywords: marine fungi, Mediterranean Sea, *Aplysina cavernicola*, *Crambe crambe*, *Phorbas tenacior*

Introduction

Oceans host a high biodiversity including the well-studied animals and plants, and the largely under-investigated microorganisms. To date, research on microorganisms has mostly focused on bacteria leaving fungi largely neglected. Marine fungi are widespread in the oligotrophic oceanic waters (Raghukumar 2017), where they are involved in the cycling of nutrients by decomposing organic matter like lignin-cellulose materials and chitin, the two most recalcitrant and abundant polymers in nature (Bongiorni *et al.* 2005; Panno *et al.* 2013; Alamgir 2017; Balabanova *et al.* 2018). Marine fungi have also been reported as pathogens in both natural and anthropogenic environments, i.e. aquaculture systems (Raghukumar 2017), although they are also involved in symbioses, as observed in marine lichens (Lipnicki 2015) or as hypothesised for seaweeds (Sakayaroj *et al.* 2010; Vohník *et al.* 2017) and animals (Raghukumar 2017).

In 1849 Desmaziéres reported the first fungus in the sea (Raghukumar 2017). Nowadays, 1,206 marine fungal species have been described, although recent estimates indicate the existence of more than 10,000 species (Jones *et al.* 2015; Pang & Jones 2017). Besides mycologists, chemists too are interested in marine fungal diversity, considering marine fungi a promising source of secondary metabolites. In 1953, the discovery of cephalosporin C, isolated from *Cephalosporium acremonium*, opened the way to the search for natural molecules isolated from marine fungi (e.g. anti-cancer, antimicrobial and anti-inflammatory compounds) (Abraham 1979; Imhoff 2016). Among marine fungi, those associated with sponges produce 28% of the novel secondary metabolites recorded to date (Imhoff 2016). Marine fungi produce a wide range of bioactive molecules that may act as chemical defence against sponges' predators, pathogens and fouling organisms while they also secrete hydrolytic enzymes that convert complex organic matter into nutrients easily accessible for sponges (Debbab *et al.* 2012). Moreover, since metabolites previously ascribed to sponges are structurally similar to those produced by the associated bacteria (Imhoff & Stöhr 2003; Henríquez *et al.* 2014), fungi, and not sponges, could be the real producers of bioactive molecules.

In order to obtain new natural products and to avoid the re-extraction of known compounds, a low-rank taxonomic identification of fungi is fundamental for the de-replication with the databases; unfortunately, to date, most of the studies on natural products provide fungal identifications at genus level only (Imhoff 2016; Reich & Labes 2017).

In the present work, we focused on the culturable mycobiota associated with three Mediterranean Demospongiae: *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior*. These three sponges are well-known for the production of secondary metabolites. Indeed, *A. cavernicola* produces bromotyrosine-derived metabolites with anti-feeding, antibacterial and cytotoxic properties. The production of these compounds varies with seasons and is mainly influenced by water temperature (Reverter at al. 2016). *C. crambe* has been widely studied since the early 1990s, for example, Uriz *et al.* (1996) pointed out that the outer layer of this animal was more toxic than the inner one. This toxicity is probably due to polycyclic guanidine alkaloids, whose anticancer activity has been demonstrated (Ternon *et al.* 2016). As for *A. cavernicola*, the production of secondary metabolites shows a seasonal fluctuation in this latter species (Becerro *et al.* 1997). Compared to the other two sponges our knowledge on the metabolome of *P. tenacior* (syn. *Anchinoe tenacior*) is still scarce, given that Casapullo *et al.* (1993, 1994a, b) recorded few pseudopeptides alkaloids, whose biological activity has not yet been demonstrated. Here we present for the first the mycobiota associated with these three Mediterranean sponges characterized by different and unique metabolomes.

Material and methods

Samples collection

The Mediterranean sponges *Aplysina cavernicola*, *Crambe crambe* and *Phorbas tenacior* (three specimens each) were collected in summer by scuba divers, in the Mediterranean Sea at Villefranche sur Mer, France (Lat: 43° 41' 31.48707839999" N, Lon: 7° 19' 12.185658623999" E), at about 25 m depth. *A. cavernicola* and *P. tenacior* were sampled on the same rock, whereas *C. crambe* was collected on a separate rock few meters apart.

Fungal isolation

Specimens were surface sterilized with ethanol 70% (for 30 sec) and serial washed (three times) in sterile seawater to eliminate debris and microorganisms not strictly associated with sponges. Different growth media and incubation temperatures were used to increase the number of fungal isolates. Sponges were homogenized (homogenizer blade Sterilmixer II - PBI International, Milan, Italy) and diluted 1:10 w v^{-1} in sterile seawater. One mL of solution was spread in each Petri dish (15 cm Ø). Three different growth media were used: solid media Sea Water Agar – SWA (sea salts mix 30 g, agar 15 g - Sigma-Aldrich, Saint Louis, USA – up to 1 L H_2O), Corn Meal Agar Sea Water - CMASW (corn meal 2 g, agar 15 g, sea salts mix 30 g -

Sigma-Aldrich, Saint Louis, USA – up to 1 L H_2O) and Gelatin Agar Sea Water – GASW (gelatin 20 g, agar 15 g, sea salts mix 30 g - Sigma-Aldrich, Saint Louis, USA – up to 1 L H_2O). Gelatin was used to mimic the composition of the organic material since sponges are rich in collagen. Three replicates for each medium and incubation temperature (15 °C and 25 °C) were performed. To prevent bacterial growth, all media were supplemented with an antibiotic mix (Gentamicin Sulfate 40 mg L^{-1}, Piperacillin and Tazobactam 11 mg L^{-1}). Plates were incubated in the dark and monitored for 30 days. Fungal strains that developed were transferred to axenic cultures and taxonomically identified.

In the case of *A. cavernicola*, due to the abundance of samples, a second isolation technique called "direct plating" was applied. The sponge specimens were sterilized and cut in pieces of 1 cm^3, which were directly plated in Petri dishes (6 cm Ø); the media, the incubation temperatures and the number of replicates were the same as previously reported.

Fungal identification

Fungi were identified using a polyphasic approach, combining morpho-physiological observation of the colonies, with molecular tools. The DNA was extracted using a NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer instructions. DNA markers were amplified using specific primers for each fungal genus. For the genus *Cladosporium*, the actin gene was amplified with primers ACT512F/ACT783R (Carbone & Kohn 1999). For *Penicillium* and *Aspergillus* genera, the β-tubulin gene was amplified using primers Bt2a/Bt2b (Glass e Donaldson 1995). For all the other genera and sterile mycelia, the Internal Transcribed Spacer (ITS) was amplified using ITS1/ITS4 (White *et al.* 1990). Sterile mycelia, whose morphological identification was not possible, underwent phylogenetic analysis. Following amplification of the large ribosomal subunit (LSU), using primer pair LROR/LR7 (Vilgalys & Hester 1990), three datasets were created: two for the orders *Pleosporales* and *Capnodiales* and one for the class *Sordariomycetes*. Alignments were generated using MEGA 7.0 and manually refined. Phylogenetic analyses were inferred using a Bayesian Inference (BI; MrBayse 3.2.2 four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC), run over 10 million generations (under GTR + Γ + I evolutionary model). The first 2,500 trees were discarded as "burn-in" (25%). Using the Sumt function of MrBayes a consensus tree was generated and Bayesian posterior probabilities (BPP) were estimated. In a second approach,

Maximum Likelihood (ML) estimate was performed using RAxML v. 8.1.2 (Stamatakis 2014) with the same substitution model (GTR + Γ + I) and 1,000 boostrap replicates. Support values from bootstrapping runs (MLB) were mapped on the globally best tree using the "-f a" option of RAxML and "-x 12345" as a random seed to invoke the novel rapid bootstrapping algorithm.

In order to recognize identical strains, isolates of the same species underwent mini-satellite screening by using minisatellite core sequence derived from the wild-type phage M-13 as a primer. Amplicons were separated on 1.5% agarose gel stained with 5 µL 100 mL^{-1} ethidium bromide; a GelPilot 1 kb plus DNA Ladder was used as reference; images were acquired with a Gel Doc 1000 System (Bio-Rad, Hercules, CA, USA) and analysed using Bionumerics 7.1 (Poli *et al.* 2016).

Strains are preserved at the *Mycotheca Universitatis Taurinensis* (MUT - http://www.mut.unito.it) of the University of Turin (Italy). Newly generated sequences were deposited in GenBank (see Table 1 and Table 2).

Results and discussion

Sponges are the first source of new marine natural products (Mehbub *et al.* 2014) and they host microorganisms, including fungi, which produce bioactive molecules. Recently, several studies started to focus on fungi associated with sponges; however, to the best of our knowledge, less than 2% of sponge species have been studied for the associated fungal communities.

Overall, 29 taxa were isolated from the three sponges: *Phorbas tenacior* hosted 19 taxa (27 isolates), followed by *Aplysina cavernicola* (24 isolates representing 13 species), and *Crambe crambe* (three species-isolates).

Influence of the isolation techniques on the number of culturable fungi isolated

The use of different isolation techniques increased the possibility of fungal isolation: even if the sponges were sampled in the same place and at the same depth, the cultivable mycobiota of each organism showed a preference for different incubation temperatures. Overall, 80.0% and 66.7% of taxa were exclusively isolated at 25 °C from *P. tenacior* and *C. crambe*, respectively (Fig. 1A). As for *A. cavernicola*, half of the taxa (50.0%) were exclusively isolated at 15 °C, 23.1% at 25 °C, while the remaining 23.1% were isolated at both temperatures. The use of different incubation temperatures for successful isolation of a high number of

sponges-associated fungi has been reported only once (Bovio *et al.* 2018). Whether an ecological explanation of this phenomenon exists is an issue that requires further studies. In our case, the main difference between *A. cavernicola* and the other two sponges (where a higher number of fungi was recorded at 25 °C) is the ecological niche: *A. cavernicola* usually grows under rocks and, being less exposed to the sun could prefer lower temperatures.

In addition, the use of different growth media also proved to be important to increase the number of isolated taxa. Fungi from *A. cavernicola* and *P. tenacior* were isolated on all the media at different percentages (Fig. 1B). Fungi from *C. crambe* grew exclusively on GASW and SWA, media specifically selected to mimic the isolation organic material and the environment. These two media were particularly effective for the isolation of *Basidiomycota*: *Irpex lacteus*, *Psathyrella candolleana* and *Schizophyllum commune* were isolated on unconventional poor media (GSWA and SWA) re-enforcing the hypothesis that marine *Basidiomycota* requires specific growth conditions (Ding *et al.* 2011).

The use of media that mimic environmental conditions is one of the factors that increase and diversify the number of cultivable fungi. In fact, it is clear that media rich in nutrients do not ensure a high fungal diversity, supporting the growth of few fast-growing fungi (Caballero-George *et al.* 2013). Noteworthy, all *Penicillium* spp. were isolated only on CMASW and GSWA, indicating that SWA can be considered as a selective medium that limit the growth of highly sporulating fungi.

The mycobiota

By means of morphological, molecular and phylogenetic approaches, all taxa were identified at the species level, with the exception of one strain (*Hypocreales* sp.) that did not grow in axenic conditions. A remarkable number of fungi (31%), belonging to *Capnodiales*, *Pleosporales* and *Sordariomycetes*, remained sterile in pure culture (Table 1) and their taxonomic placement was inferred with phylogenetic analyses (Supplementary materials Figs. 3-5). The three sterile *Basidiomycota* were further analysed by Poli *et al.* (2018). A detailed identification of fungi is fundamental for the following reasons: i) to compare the fungal community among different substrates; ii) to perform ecological studies; iii) to exploit these organisms for the production of secondary metabolites and/or other biotechnological purposes; iv) to handle the organisms in accordance with the latest biosafety regulations. Moreover, as reported by Paz *et al.* (2010), the systematic identification of fungi, frequently underestimated, increases the reliability of the work. The fungal diversity

associated with the three sponges was mainly assigned to *Ascomycota* (89.7%), whereas *Basidiomycota* represented only the 10.3% of the taxa, which is not unusual in marine environments (Suryanarayanan 2012; Jones *et al.* 2015). Possibly, marine *Ascomycota* are involved in processes that in terrestrial habitats are carried out by *Basidiomycota*, such as the degradation of lignocellulosic materials (Panno *et al.* 2013; Raghukumar 2017; Balabanova *et al.* 2018) or hydrocarbons (Garzoli *et al.* 2015a; Bovio *et al.* 2017). However, it must be considered that *Basidiomycota* could be poorly retrieved in the marine environment due to not selective isolation techniques (Ding *et al.* 2011).

The most represented genera, in terms of species, were *Penicillium* (eight species) and *Cladosporium* (five species), two genera commonly found in association with sponges that are among the most studied for the isolation of new natural products (Imhoff 2016).

Thanks to the low-rank classification, it was possible to compare the fungal species retrieved in the present study with those already reported in marine ecosystems: 35.7% and 53.6% of the species were detected for the first time either in the marine environment or in association with sponges (Table 1). Considering the mycobiota of each sponge, two out of three of the studied animals hosted a specific fungal community (Fig. 2), with 85.0% and 61.5% of the species exclusive, respectively, to *P. tenacior* and *A. cavernicola*. According to Li & Wang (2009), these fungi, strictly retrieved only on one sponge, can be defined as "sponge specialists".

With regard to *C. crambe*, only one species out of three was "sponge specialist". This species, *Kernia geniculotricha*, reported here for the first time from both a sponge and in the marine environment, is a coprophilous fungus on land ecosystems (Seth 1968; Saxena & Mukerji 1970).

As for the common species (Fig. 2), *Cladosporium pseudocladosporioides* was shared among the three sponges and can be defined "sponge-generalist" due to its ubiquity (Li and Wang 2009). The same species was recorded in the Mediterranean Sea in an oil-spilled site, in association with the red alga *Asparagopsis taxiformis* (Garzoli *et al.* 2015b; Bovio *et al.* 2017) and in some Atlantic sponges (Bovio *et al.* 2018). *Penicillium citrinum* was shared by *A. cavernicola* and *C. crambe*, and was previously found in the Mediterranean sponge *Psammocinia* sp. (Paz *et al.* 2010). The retrieval of this species is not surprising since it is ubiquitous in Mediterranean and Oceanic ecosystems (see Table 1 for references).

A. cavernicola and *P. tenacior* shared additional four species (Fig. 2): *Cladosporium cladosporioides* and *Penicillium brevicompactum* already reported in association with sponges and *Neosetophoma samararum* and *Cladosporium perangustum* firstly isolated from a sponge (see Table 1 for references). Being present in more than one species of sponge, these fungi can be defined as sponge-associated, according to Li & Wang (2009). By applying the classification of Li & Wang (2009), in the attempt to discriminate between fungi not strictly associated with sponges from those closely associated, it was clear that the three sponges species host a specific mycobiota. This idea is supported by a mini-satellite analysis: isolates of the same species retrieved from different sponges did not belong to the same strain (Supplementary materials Figs. 6-11). There were two exceptions: *C. pseudocladosporioides* (MUT 3559 and MUT 3580) and *C. cladosporioides* (MUT 3540 and MUT 3571) that were found both on *A. cavernicola* and *P. tenacior*.

The fungal-sponge specificity is still debated. Metagenomic studies showed the overlapping of sponges-fungal OTUs with those of water samples (Naim *et al.* 2017); on the contrary, Jin *et al.* (2014) demonstrated a specific association between fungi and sponges.

Mycobiota *vs* metabolome

The three Mediterranean sponges hosted different mycobiotas; this could be due to several factors, including the metabolites produced by each sponge. *C. crambe* hosts the lowest biodiversity, with only three species. Actually, this sponge is well known for the production of toxic compounds (including polycyclic guanidine alkaloids), which are concentrated in the outer layer (Uriz *et al.* 1996; Ternon *et al.* 2016), making this organism scarcely colonisable by bacteria (Becerro *et al.* 1997). In fact, *C. crambe* belongs to the "low-microbial-abundance sponges" characterized by an average amount of bacteria per g of sponge-wet weight lower than that of seawater (Sipkema *et al.* 2015). In addition, in comparison to seawater, also the bacterial diversity within the sponge is lower; different studies reported that the bacterial community of *C. crambe* is dominated by *Betaproteobacteria*, that on average comprise 85% of the population (Croué *et al.* 2013; Gantt *et al.* 2017). By using a culturomic approach, Öztürk *et al.* (2013) obtained only 107 bacterial isolates from *C. crambe,* despite the use of 16 isolation media and several sponge specimens. Indeed, the toxic compounds produced by this sponge could strongly select the fungal community able to grow on the sponge and/or inhibit the fungal growth in the isolation plates. From an ecological point of view, these compounds, once

released, have teratogen effects on ascidian embryos, supporting the hypothesis that encrusting sponges like *C. crambe*, use toxic metabolites to mediate the colonization of new habitats (Ternon *et al.* 2016).

The production of bromotyrosine-derived compounds reported in *A. cavernicola* (Reverter at al. 2016) could contribute to the recruitment of the fungal community. In fact, bromotyrosine-derivatives were isolated with the aim of finding new antibiotics of marine origin and they proved to be active against several bacteria and pathogenic fungi (Peng *et al.* 2005). Besides, several bacteria isolated from *A. cavernicola* produced antimicrobial compounds (Hentschel *et al.* 2001) stimulating once more the question of whether sponges or their associated microorganisms are the real producers of antimicrobial compounds.

P. tenacior displayed the highest diversity and the highest percentage of exclusive taxa (85%). However, due to the lack of information about its metabolome, no hypothesis on the ability of this sponge to select its mycobiota can be drawn. On the contrary, the low biodiversity of the bacterial community might be the result of insufficient or unsuitable isolation techniques (Dupont *et al.* 2013).

The mycobiota of *A. cavernicola*, *C. crambe* and *P. tenacior* compared with other sponges

Despite the Mediterranean Sea is a hot spot of biodiversity for sponges (Jakson *et al.* 2015), only a few of them have been studied for their associated mycobiota using a cultural-dependent approach. In comparison to the three sponges studied in this investigation, a higher number of fungi was reported in two Mediterranean Demospongiae: *Psammocinia* sp. (85 taxa) (Paz *et al.* 2010) and *Suberites domuncula* (81 strains) (Proksch *et al.* 2008); however, only a few taxa were identified at the species level, thus preventing a proper comparison with our results. On the other hand, several sponges, worldwide, hosted a low fungal diversity; for instance, a total of 22 fungal species was found in seven sponges in the Red Sea (Egypt) (Sayed *et al.* 2016) and 24 fungal genotypes were isolated from ten Antarctic sponges (Henríquez *et al.* 2014). Analogously to what we found in *C. crambe*, the Pacific sponge *Myxilla incrustans* hosted only two fungal taxa; the authors explained this phenomenon as a consequence of the habitat where the sponge was collected since the same species displayed a higher number of fungi when sampled in other places (Pivkin *et al.* 2006). Considering that the fungal community of *C. crambe* is reported for the first time in the present study, it is not possible to achieve a similar conclusion; further studies on different specimens would be necessary to better understand the stability of the mycobiota inhabiting this sponge. This has been done for bacteria, in

fact *C. crambe* was collected in clean and polluted sites in Spain (Mediterranean Sea), and the bacterial community appeared to be stable and dominated by *Betaproteobacteria* (Gantt *et al.* 2017). Interestingly, the same community was observed on specimens of *C. crambe* sampled 150 km apart, demonstrating the high specificity for the substrate (Croué *et al.* 2013; Gantt *et al.* 2017).

The diversity of fungi inhabiting sponges might be influenced by several factors. More studies on the same sponge species collected in different sites would be necessary; however, the sponge species (with its metabolites) seems to be one of the driving factors determining the microbiome structure. Other factors, such as the sponge structure, more or less accessible to fungi, can play key roles in shaping the sponge mycobiota (Pivkin *et al.* 2006).

Fungi from *A. cavernicola* isolated by direct plating

For the isolation of fungi from *A. cavernicola* a second isolation method, the direct plating was applied. This was not possible for *C. crambe* and *P. tenacior* since, being encrusting sponges and living in a close association with the rocky substrate, their collection was particularly hard. In addition, due to the hard structure of *C. crambe* and *P. tenacior*, it was not possible to separate the outer layer from the inner one.

Overall, considering both isolation techniques, 43% of the fungal community of *A. cavernicola* was isolated by direct plating; no overlap was observed between the taxa isolated with the two methods. Interestingly, as for the fungi recorded by homogenization of sponges' tissues, more taxa (60%) were isolated at 15 °C than at 25 °C (40%). The use of different media proved fundamental to increase the number of cultivable fungi: each fungal species was isolated only on one medium (Table 2). Of further interest was that half of the fungal community isolated by direct plating was represented by sterile mycelia (Table 2), and identified by phylogenetic analysis (Supplementary materials Figs. 3-4).

Ascomycota exclusively represented the mycobiota of *A. cavernicola* isolated by direct plating; the genera reported as the most frequent using the homogenization, (*Penicillium* spp.) were either absent or poorly represented, with only one species for the genus *Cladosporium*. The most frequent genus was *Rosellinia* (two species), firstly found within sponges but already documented in marine ecosystems (mangroves plants and/or soils) (Chareprasert *et al.* 2012). Of the ten taxa (Table 2) isolated by direct plating four species (*Sordaria fimicola, Preussia terricola, Rosellinia limonispora* and *Sarocladium glaucum*) were reported here

for the first time from marine sponges and for *S. glaucum* this was the first record from the marine environment.

Conclusion

The Mediterranean sponges *A. cavernicola*, *C. crambe* and *P. tenacior* host a specific mycobiota with only one species shared between them. The mini-satellite analysis confirmed the uniqueness of the sponge mycobiota: despite the four fungal species shared by *A. cavernicola* and *P. tenacior*, only two strains were common to the two sponges. Overall, the sponge, with its metabolome, may shape the microbial community; this was particularly clear for *C. crambe*, a sponge that produces several toxic metabolites, with one of the lowest fungal diversity ever reported in marine sponges. A total of 11 and 17 species were reported for the first time either in the marine environment or in association with sponges. Finally, more than 80 newly generated sequences were obtained and deposited in GenBank, contributing significantly to the molecular data related to marine fungi. In conclusion, this work improves our knowledge of the culturable fungal community inhabiting the Mediterranean Sea and, thanks to species-level classification, lays the foundation for further chemical study of their metabolites.

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Fig 1. Influence of the isolation techniques on the number of culturable fungi isolated from *A. cavernicola*, *C. crambe* and *P. tenacior*: **A,** influence of incubation temperatures; **B,** influence of growth media.

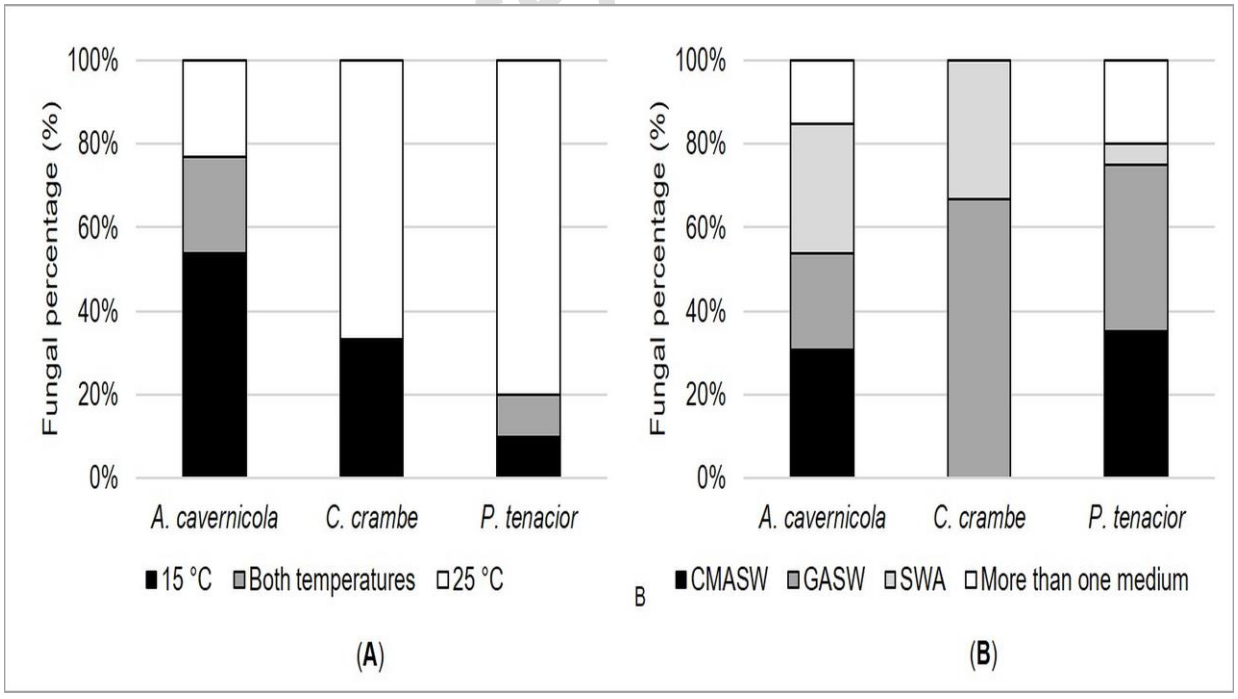

Fig 2. Number of exclusive and common fungal taxa in the three Mediterranean sponges *A. cavernicola*, *C. crambe* and *P. tenacior*.

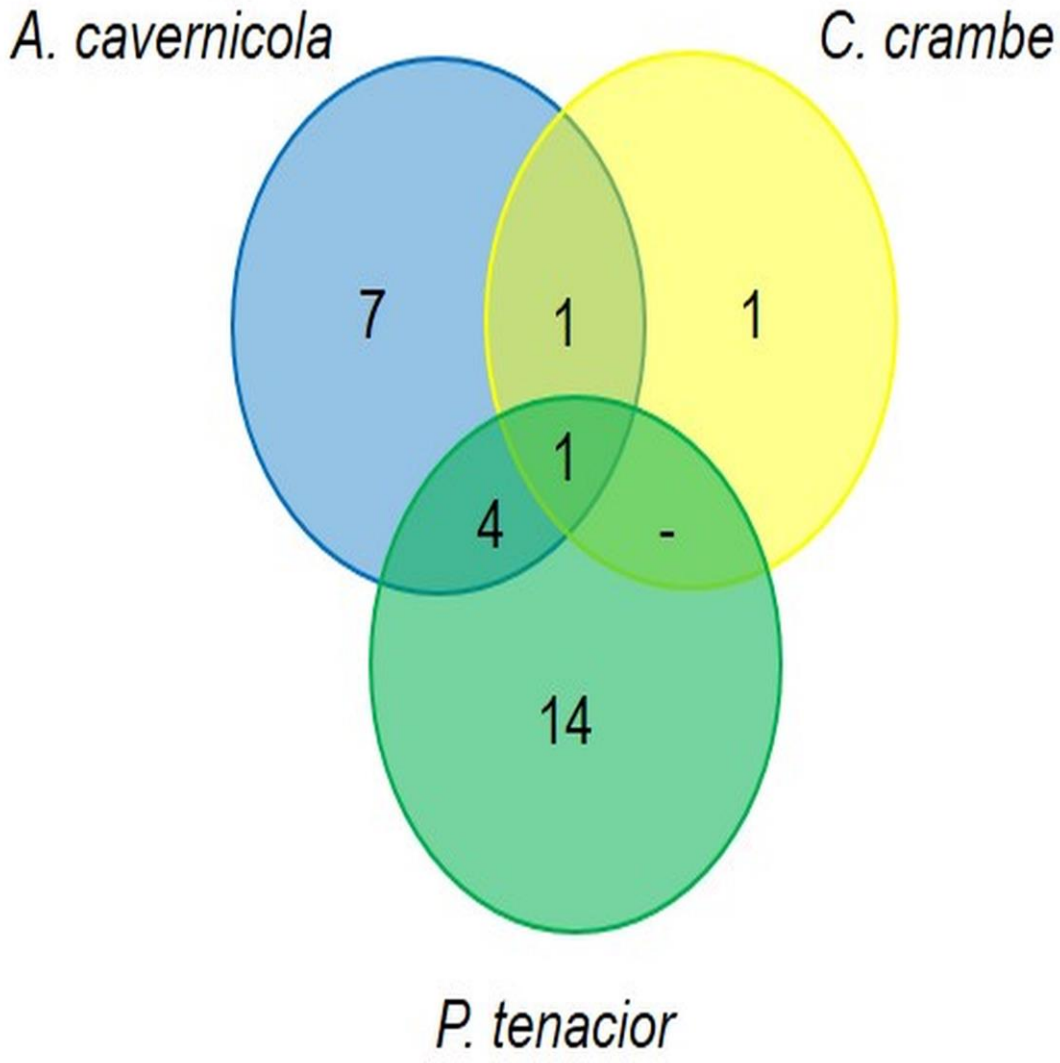

Table 1. Fungal taxa isolated from *A. cavernicola* (AC), *C. crambe* (CC) and *P. tenacior* (PT) and their incubation conditions. First record (FR) and species already found in marine environment (MA) and associated with sponges (SP) are reported. GenBank accession numbers are provided for each marker used.

MUTCODE	Fungal species	Isolation media			Incubation temperature		Sponge species	Report		GenBank accession number			
		CMA SW	GA SW	SWA	15 °C	25 °C		MA	SP	ITS	LSU	ACT	TUB
	Ascomycota												
3035	*Arthrinium arundinis**	x			x		PT	[2], [5], [4]	[26]	MG980573	MG980403		
3393	*Aspergillus protuberus*		x			x	PT	[11], [7]	FR				MH047310
3048	*Beauveria brongniartii*		x			x	PT	[7]	FR	MG980574			
3040	*Cladosporium cladosporioides*			x	x		AC	[1], [2], [5], [7], [19], [21], [22], [25]	[15], [14], [13], [12], [20], [8], [9]			MH047331	
3540				x	x		AC					MH047322	
3542				x	x		AC					MH047323	
3549			x			x	AC					MH047324	
3551				x		x	AC					MH047325	
3050			x			x	PT					MH047329	
3562				x	x		PT					MH047326	
3563			x			x	PT					MH047327	
3564			x		x		PT					MH047328	
3571		x				x	PT					MH047330	
3250	*Cladosporium delicatulum*			x	x		AC	FR	FR			MH047332	
3292	*Cladosporium perangustum*			x	x		AC	[25]	FR			MH047334	
3397		x				x	PT					MH047335	
3553	*Cladosporium pseudocladosporioides*		x			x	AC	[5]	[9]			MH047336	
3554		x				x	AC					MH047337	

3555		x				x	AC					MH047338	
3556				x		x	AC					MH047339	
3557			x		x		AC					MH047340	
3558			x		x		AC					MH047341	
3559		x				x	AC					MH047342	
3580				x		x	PT					MH047343	
3587		x				x	PT					MH047344	
3499			x		x		CC					MH047345	
3459	*Cladosporium ramotenellum*		x			x	PT	[22]	FR			MH047346	
3460	*Epicoccum nigrum*	x		x		x	PT	[5]	[17], [9]	MG980576			
3463							PT			MG980577			
2975	*Eutypella scoparia*	x				x	AC	[23]	[16]	MG980578			
-	Hypocreales sp.*		x		x		PT	-	-				
3058	*Kernia geniculotricha*			x		x	CC	FR	FR	MG980579			
3468	*Lecanicillium antillanum**			x	x		PT	FR	FR	MG980580	MG980404		
3045	*Neosetophoma samararum**			x	x		AC	[7]	FR	MG980581	MG980405		
3046		x				x	PT			MG980582	MG980406		
3471	*Penicillium brevicompactum*	x				x	PT	[1], [2], [3], [5], [7], [19], [22]	[3], [6], [10], [15], [31]				MH047313
3501		x			x		AC						MH047311
3503		x				x	AC						MH047312
3498	*Penicillium catenatum*	x				x	PT	FR	FR				MH047314
3297	*Penicillium citrinum*	x				x	AC	[4], [5], [7], [11], [21], [22], [24], [18], [30]	[3]				MH047316
3500			x			x	CC						MH047317
3560	*Penicillium corylophilum*		x		x		AC	[19], [28]	[15]	MG980583			

3561			x			x	AC					MH047318
3485	*Penicillium glabrum*		x			x	PT	[7], [19], [28], [18]	[3], [17]			MH047319
3299	*Penicillium murcianum*	x			x		AC	FR	FR			MH047320
3486	*Penicillium cinereoatrum*	x				x	PT	-	-			MH047315
3495	*Penicillium steckii*	x				x	PT	[4], [22], [18]	[3], [6]			MH047321
3038	*Torula herbarum**			x	x		PT	[1], [27]	FR	MG980592	MG980414	
3036	*Uwebraunia dekkeri**		x			x	PT	FR	FR	MG980594	MG980415	
2971	*Virgaria nigra*		x		x		AC	FR	FR	MG980595		
3034	*Xylaria badia**		x			x	PT	FR	FR	MG980596	MG980416	
	Basidiomycota											
2966	*Irpex lacteus**			x	x		AC	FR	[9]	MF098695	MF115837	
3033	*Psathyrella candolleana**		x			x	PT	FR	FR	MG980585	MG980408	
3019	*Schizophyllum commune**		x		x		AC	[2], [7]	[6]	MF098694	MF115836	

*Sterile mycelia

[1] Panno et al., 2013, [2] Gnavi et al., 2017, [3] Paz et al., 2010, [4] Raghukumar, 2017, [5] Bovio et al., 2017, [6] Gao et al., 2008, [7] Jones et al., 2015, [8] Henríquez et al., 2014, [9] Bovio et al., 2018, [10] Passarini et al., 2013, [11] Garzoli et al., 2015a, [12] San-Martin et al., 2005, [13] Manriquez et al., 2009, [14] Rozas et al., 2011, [15] Pivkin et al., 2006, [16] Bolaños et al., 2015, [17] Wiese et al., 2011, [18] Rämä et al., 2017, [19] Oren and Gunde-Cimerman, 2012, [20] Sayed et al., 2016, [21] Raghukumar and Ravindran, 2012, [22] Zajc et al., 2012, [23] Ciavatta et al., 2008, [24] Suryanarayanan, 2012, [25] Liu et al., 2017, [26] Wang et al., 2015, [27] Blunt et al., 2014, [28] Gomes et al., 2008, [29] Rämä et al., 2014, [30] Debbab et al., 2012, [31] El-Gendy et al., 2018, [32] Garzoli et al., 2015b.

Table 2. Fungal taxa isolated from *A. cavernicola* by direct plating and their incubation conditions. First record (FR) and species already found in marine environment (MA) and associated with sponges (SP) are reported. GenBank accession numbers are provided for each marker used.

MUTCODE	Fungal taxa	Isolation media			Incubation temperature		Report		GenBank accession number		
		CMASW	GASW	SWA	15 °C	25 °C	MA	SP	ITS	LSU	ACT
3236	*Chaetomium globosum*		x		x		[2], [4], [7], [5], [13], [18], [14], [19]	[15]	MG980575		
3271	*Cladosporium halotolerans*			x	x		[5], [11], [17]	[9]			MH047333
2961	*Preussia terricola**		x			x	[12]	FR	MG980584	MG980407	
3041	*Rosellinia limonispora*	x			x		FR	FR	MG980586	MG980409	
2970	*Rosellinia* sp.*	x				x	-	-	MG980587	MG980410	
3643	*Sarocladium glaucum**			x	x		FR	FR	MG980588	MG980411	
3056	*Scopulariopsis* sp.*			x	x		-	-	MG980589	MG980412	
3064	*Sordaria fimicola**		x			x	[4]	FR	MG980590	MG980413	
3308	*Stachybotrys chartarum*	x			x		[5], [7], [8], [11], [20]	[1], [6]	MG980591		
3313	*Trichoderma atroviride*			x		x	[7], [11], [18]	[3], [10], [16]	MG980593		

*Sterile mycelia

[1] Ma et al., 2015, [2] Gnavi et al., 2017, [3] Paz et al., 2010, [4] Raghukumar, 2017, [5] Bovio et al., 2017, [6] Li et al., 2014, [7] Jones et al., 2015, [8] Kis-Papo et al., 2001, [9] Bovio et al., 2018, [10] Passarini et al., 2013, [11] Garzoli et al., 2015a, [12] Costello et al., 2001, [13] Suryanarayanan, 2012, [14] Gomes et al., 2008, [15] Pivkin et al., 2006, [16] Bolaños et al., 2015, [17] Zajc et al., 2012, [18] Rämä et al., 2017, [19] Oren and Gunde-Cimerman, 2012, [20] Raghukumar and Ravindran, 2012.