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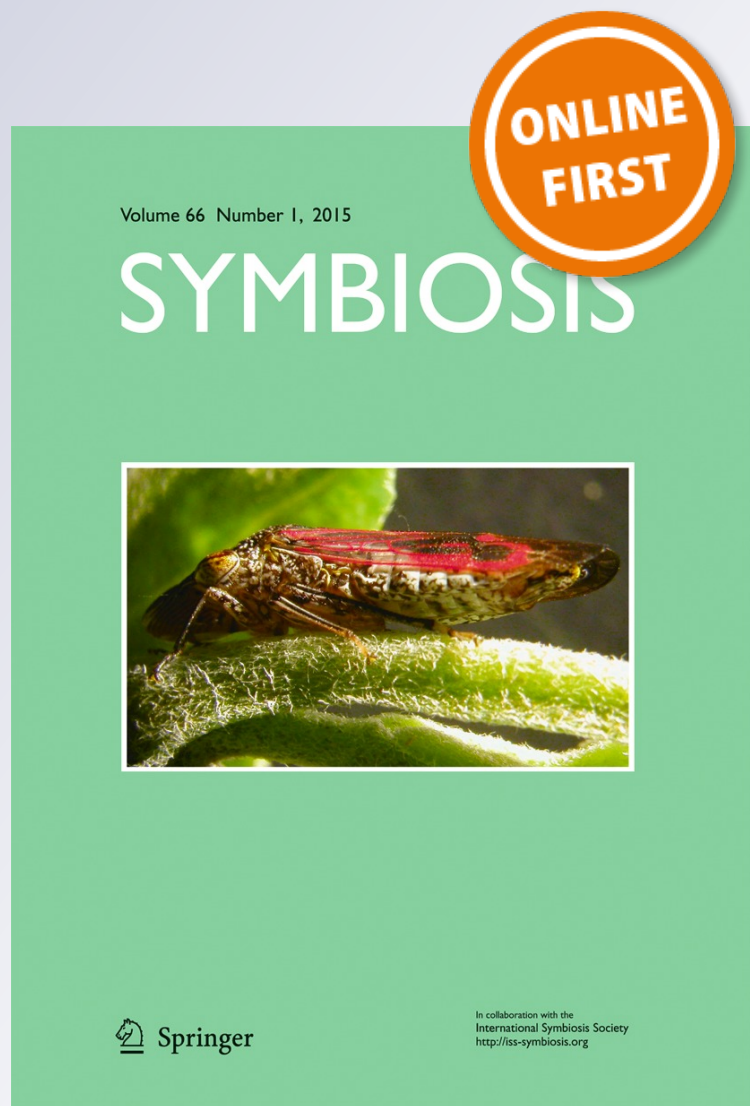
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**Symbiosis**

ISSN 0334-5114

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DOI 10.1007/s13199-015-0339-x



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# Diversity of fungi isolated from three temperate ascidians

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**Abstract** Ascidians are known to harbor diverse and host-specific bacterial and archaeal communities in their tunic. However, to date, only one ascidian species has been investigated to assess symbiotic relationships with fungi and the extent of their diversity. In this study, we isolated and identified 37 strains of fungi in association with three common ascidian species in the NW Mediterranean Sea: *Cystodytes dellechiajei*, *Didemnum fulgens*, and *Pycnoclavella communis*, and 15 additional strains from concentrated seawater samples collected around the animals. Most of the isolated fungi were classified within four orders: Eurotiales (predominantly *Penicillium* spp.), Pleosporales, Hypocreales (predominantly *Trichoderma* spp.), and Capnodiales (*Cladosporium* spp.). Three additional fungal isolates from *C. dellechiajei* and *D. fulgens* belonged to the orders Helotiales, Phylachorales and Microascales, and matched to well-known plant and human pathogens (*Botrytis cinerea*, *Plectosphaerella cucumerina* and *Scopulariopsis brevicaulis*). Host-specificity of ascidian-associated fungi was not apparent and thus the significance of ascidian-fungal associations for ascidian

wellbeing and their possible ecological roles remain unknown.

**Keywords** Fungus · Mediterranean Sea · ITS · Sea-squirt · Tunicata · Pathogens

## 1 Introduction

Invertebrate-bacterial associations and, to a lesser degree, invertebrate-archaeal associations have been widely described in the literature (e.g., Mouchka et al. 2010; Webster and Taylor 2012; Erwin et al. 2014). Most of these studies have also demonstrated a high degree of specificity between the invertebrate host and at least a core community of its symbionts (Taylor et al. 2004; Ainsworth et al. 2010, 2015; Schmitt et al. 2012; Easson and Thacker 2014; Erwin et al. 2014). Moreover, some of these associations are obligate, in that both the host and the symbiont depend on each other for their long-term survival (Ainsworth et al. 2010; Thacker and Freeman 2012). Other potential invertebrate-microbial associations include symbioses with eukaryotes (e.g., dinoflagellates in corals; reviewed in Rowan 1998) and fungi (reviewed in Yarden 2014). When compared with other reported symbiotic relationships, the latter has been surprisingly understudied and has mostly focused on corals and sponges. To date, we know that coral-associated fungi are prevalent and that in at least a few species these associations are host-specific rather than environmentally determined (e.g., Amend et al. 2012). In sponges, hundreds of fungal strains have been isolated and identified as either marine or ubiquitous taxa (Höller et al. 2000; Morrison-Gardiner 2002; Li and Wang 2009; Menezes et al. 2010; Paz et al. 2010; Wiese et al. 2011) with several fungal strains producing previously unknown secondary metabolites (Höller et al. 2000; Paz et al. 2010; Wiese et al. 2011;

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Koch et al. 2014). However, evidence for host-specificity is still rare, and has only been demonstrated in a few cases (Li and Wang 2009).

Ascidians or sea-squirts (Chordata, Tunicata) are conspicuous benthic sessile invertebrates found all around the world (Shenkar and Swalla 2011). Ascidians are especially abundant in hard bottom rocky substrates and attached to artificial substrates, such as docks and aquaculture cages (Lambert 2001; Carman et al. 2010). With few exceptions, ascidians are filter-feeding organisms with an abundance and diversity of species and functions that render them critical to healthy ecosystem functioning (Lambert 2005). As sessile invertebrates, ascidians are also easy prey for predators and thus have developed a diverse array of defensive secondary metabolites (Pisut and Pawlik 2002; Tarjuelo et al. 2002; López-Legentil et al. 2006b). In fact, ascidians are well-known producers of marine natural products (Paul et al. 2011; Blunt et al. 2014, 2015) and several of these secondary metabolites have also shown interesting pharmaceutical properties, especially as anti-cancer drugs (Erwin et al. 2010). Although the importance of many of these compounds is well established, to date there is little information about exactly who produces them, since ascidians are known to establish complex symbiotic associations with a wide range of bacteria and archaea (Tait et al. 2007; Erwin et al. 2013, 2014; Tianero et al. 2015). Another possible source of these bioactive compounds are fungal associates (Xin et al. 2007; Montenegro et al. 2012), but this potentially important symbiotic association between ascidians and fungi has been largely understudied. To the best of our knowledge, only one previous study has described the diversity of culturable fungi associated with an ascidian and has found that the colonial species *Didemnum* sp. (sampled in Brazil) hosted a higher diversity of filamentous fungi than the investigated, sympatric sponge species (i.e., *Mycale laxissima*, *Amphimedon viridis* and *Dragmacidon reticulata*; Menezes et al. 2010).

In this study, we targeted three colonial ascidians commonly found in rocky bottom habitats of the NW Mediterranean Sea: *Cystodytes dellechiajei*, *Didemnum fulgens*, and *Pycnoclavella communis*. The species *C. dellechiajei* is distributed worldwide in tropical and temperate waters and exhibits high phenotypic plasticity with many color morphs and chemotypes (López-Legentil et al. 2005; López-Legentil and Turon 2005). The purple morph in particular, is one of the most abundant morphotypes in the NW Mediterranean Sea and produces a set of secondary metabolites classified as pyridoacridine alkaloids (López-Legentil et al. 2005; Bontemps et al. 2010; Bry et al. 2011). Several of these metabolites are known to have anti-predatory (López-Legentil et al. 2006b), antimicrobial (Bontemps et al. 2010) and anti-tumoral (Martínez-García et al. 2007a) activities. In addition, the production of the four most common pyridoacridine alkaloids (namely shermilamine B, kuanoniamine D, and their

deacetylated forms) in the purple morph were quantified over time and shown to lack statistically significant variation, although minimum values were consistently recorded in late summer after the reproductive period of the species (López-Legentil et al. 2006a). Finally, *C. dellechiajei* is also known to host a wide array of bacterial symbionts (Martínez-García et al. 2007b), including oxygenic phototrophs containing chlorophylls *a*, *b*, *c*, and *d* (Martínez-García et al. 2011).

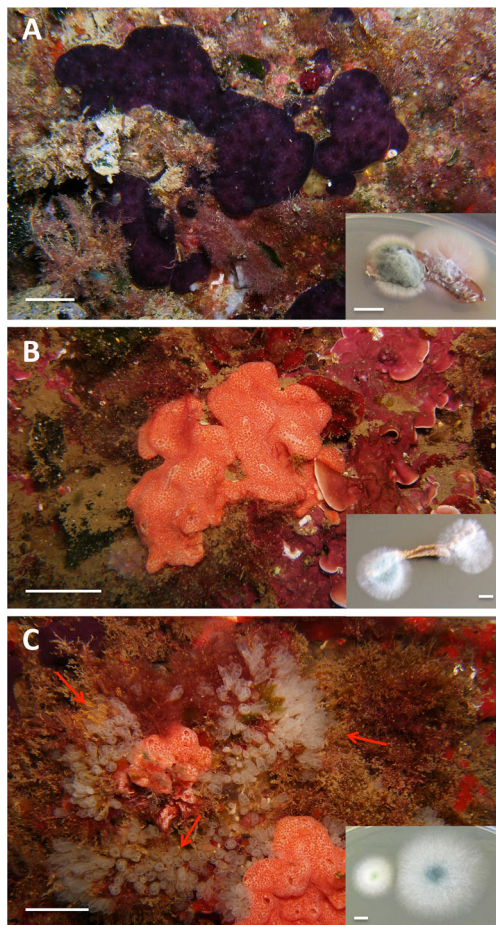
*D. fulgens* exhibits a patchy distribution along the NE coast of the Iberian Peninsula, reaching densities of >12 colonies per meter square in some areas (López-Legentil et al. 2013). This species is also known to harbor a stable bacterial community in its tunic with at least some of these symbionts being transferred to the larvae (López-Legentil et al. 2015). *D. fulgens* chemistry has not yet been investigated, however this species is likely to produce bioactive secondary metabolites given the prevalence of bioactive species in this ascidian genus (Blunt et al. 2015; and earlier reviews). Finally, *P. communis* is one of the most common colonial ascidians reported along the western Mediterranean shores (Pérez-Portela et al. 2007). Related species in the genus *Pycnoclavella* are known to produce bioactive secondary metabolites (Appleton et al. 2002; Appleton and Copp 2003) and *P. communis* in particular appears to be chemically defended against predators (Pérez-Portela and Turon 2007).

The goal of this study was to determine whether culturable fungi could be successfully isolated from three common ascidian species in the NW Mediterranean Sea, and if so, assess their diversity by DNA sequence analysis of the nuclear ribosomal internal transcribed spacer (ITS) region. To achieve these objectives, fungi were isolated and identified from three ascidian species (*C. dellechiajei*, *D. fulgens*, and *P. communis*) collected at three time points (May 2012, Oct 2012, May 2013) and from triplicate ambient seawater samples (collected May 2013).

## 2 Material and methods

### 2.1 Sample collection

Samples were collected from L'Escala, Spain ('La Depuradora': 42° 7' 29" N, 3° 7' 57" E; NW Mediterranean Sea) at depths <12 m during three collection trips carried out on May 18, 2012 (seawater temperature 16 °C), October 4, 2012 (seawater temperature 18 °C) and May 10, 2013 (seawater temperature 15 °C). Three species of colonial ascidians were targeted: the purple morph of *Cystodytes dellechiajei* (Della Valle, 1877), *Didemnum fulgens* (Milne-Edwards, 1841), and *Pycnoclavella communis* Pérez-Portela, Duran and Turon, 2007 (Fig. 1). Ascidian and seawater samples (1L) were collected by SCUBA, brought to the surface and immediately processed. The exterior of each ascidian species



**Fig. 1** In situ photographs of the colonial ascidians *Cystodytes dellechiaiei* (a), *Didemnum fulgens* (b), and *Pycnoclavella communis* (c; indicated by arrows) in L'Escala, Spain (NW Mediterranean Sea). Scale bars: 2 cm. Insets show representative fungi growing from tunic slices of *C. dellechiaiei* (a) and *D. fulgens* (b), and the whole zooid (including the tunic) of *P. communis* (c; zooids are buried under the growing fungi). Scale bar: 1 cm

was rinsed and gently dried to remove loosely attached surface organisms, then placed into a sterile petri dish for dissection on site. For *C. dellechiaiei* and *D. fulgens*, each colony was dissected in 2 to 3 thin bands (1 to 2 mm wide, 2 to 3 cm in length, < 0.5 cm in height) that included both tunic tissue and zooids. In addition, three zooids of *C. dellechiaiei* were isolated from the tunic and plated separately. For *P. communis*, each colony was dissected into 6 zooid plus tunic sections. Different dissection methods were employed to accommodate differences in zooid size (>1 mm in *C. dellechiaiei*, <1 mm in *D. fulgens*, 2–3 mm in *P. communis*) and arrangement (*C. dellechiaiei* and *D. fulgens* zooids are embedded in a common tunic, while *P. communis* zooids are connected by tunic strings and tightly surrounded by individual tunics). All ascidian dissections were immediately placed on culture plates (see below) and into an insulated cooler for transport to the laboratory (ca. 2 h transit time). On the final collection trip (May 10, 2013), we also collected triplicate ambient seawater

samples (1 L each). Seawater samples were collected in close proximity of the ascidians (<4 m) in 3 sterile Nalgene bottles (one per replicate), concentrated on 0.2  $\mu\text{m}$  polycarbonate filters (Whatman) and placed upside down (i.e., filtrate in direct contact with culture media) on 3 separate culture plates (see below).

## 2.2 Fungal isolation

Tunic bands and zooids dissected from the different ascidians and seawater filtrates were used to inoculate culture plates containing malt extract agar (Merckoplate<sup>®</sup>, Merck, Kenilworth, NJ, USA) to grow a wide spectrum of fungi and amended with the antibacterial chemicals chloramphenicol and gentamycin. Plates were incubated in the dark (to mimic conditions in the tunic) for 6 days at room temperature. Once growth was visible, different fungal morphotypes (based on color and shape differences) were isolated and streaked to purity on separate culture plates. Fungi were then allowed to grow for 7 days before a sample of each pure culture was collected for DNA extraction.

## 2.3 DNA extraction and ITS amplification

DNA was extracted using the Animal Tissue Protocol, DNeasy<sup>®</sup> Blood and Tissue kit (Qiagen<sup>®</sup>) and used as template for PCR amplification of fungal internal transcribed spacer (ITS) gene sequences using the forward primer ITS1 and the reverse primer ITS4 (White et al. 1990). The thermocycler program consisted of an initial denaturing step at 94  $^{\circ}\text{C}$  for 6 min, 35 amplification cycles (denaturing at 94  $^{\circ}\text{C}$  for 30 s, annealing at 55  $^{\circ}\text{C}$  for 30 s and extension at 72  $^{\circ}\text{C}$  for 40 s), and a final extension at 72  $^{\circ}\text{C}$  for 7 min, performed on a PCR System 9700 (Applied Biosystems). Cleaning and sequencing reactions were carried out by Macrogen, Inc. (Seoul, Korea) and consensus sequences (see below) were deposited in the GenBank database (Table 1).

## 2.4 DNA sequence and phylogenetic analyses

Raw sequence reads were processed in Geneious v. 8 (Kearse et al. 2012) by aligning forward and reverse reads to yield final consensus sequences. Each consensus sequence was then analyzed using the BLASTn tool from GenBank to assess taxonomic affiliations. For phylogenetic analyses, all ascidian-derived sequences were aligned using Clustal W v. 2 (Larkin et al. 2007) with a gap opening penalty of 24 and a gap extension penalty of 4. A phylogenetic tree was built using the neighbor-joining (NJ) method implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Tamura et al. 2013). Data were re-sampled using 1000 bootstrap replicates (Felsenstein 1985).

**Table 1** Fungal taxa isolated from the colonial ascidians *Cystodytes dellechiajei*, *Didemnum fulgens*, and *Pycnoclavella communis* and from ambient seawater samples

Ascidian species	Col. date	Fungal order	Potential species	Code	Acc. no.	
<i>Cystodytes dellechiajei</i>	18-May-12	Eurotiales	<i>Penicillium</i> sp.	CD-1	KT121502	
	18-May-12	Eurotiales	<i>Aspergillus</i> sp.	CD-3	KT121532	
	4-Oct-12	Pleosporales	<i>Epicoccum nigrum</i>	CD-1b	KT121518	
	4-Oct-12	Pleosporales	<i>Alternaria</i> sp.	CD-2b	KT121535	
	4-Oct-12	Pleosporales	<i>Phoma</i> sp.	CD-3b	KT121495	
	4-Oct-12	Eurotiales	<i>Penicillium</i> sp.	CD-4b	KT121501	
	10-May-13	Phylachrocales	<i>Plectosphaerella</i> sp.	1CD-1	KT121494	
	10-May-13	Capnodiales	<i>Cladosporium</i> sp.	1CD-2	KT121528	
	10-May-13	Hypocreales	<i>Fusarium</i> sp.	1CD-3	KT121517	
	10-May-13	Eurotiales	<i>Penicillium brevicompactum</i>	2CD-1	KT121512	
	10-May-13	Eurotiales	<i>Penicillium</i> sp.	3CD-1	KT121509	
	10-May-13	Helotiales	<i>Botrytis cinerea</i>	3CD-2	KT121529	
<i>Didemnum fulgens</i>	18-May-12	Eurotiales	<i>Penicillium</i> sp.	DF-2	KT121500	
	18-May-12	Capnodiales	<i>Cladosporium</i> sp.	DF-3	KT121520	
	18-May-12	Eurotiales	<i>Penicillium</i> sp.	DF-4	KT121499	
	4-Oct-12	Botryosphaeriales	<i>Microdiplodia</i> sp.	DF-1B	KT121516	
	10-May-13	Microascales	<i>Scopulariopsis</i> sp.	1DF-1	KT121492	
	10-May-13	Eurotiales	<i>Penicillium brevicompactum</i>	1DF-2	KT121515	
	10-May-13	Capnodiales	<i>Cladosporium</i> sp.	1DF-3	KT121527	
	10-May-13	Eurotiales	<i>Penicillium brevicompactum</i>	1DF-4	KT121514	
	10-May-13	Hypocreales	<i>Trichoderma</i> sp.	2DF-1	KT121488	
	10-May-13	Eurotiales	<i>Penicillium brevicompactum</i>	2DF-2	KT121511	
	10-May-13	Hypocreales	<i>Clonostachys</i> sp.	2DF-3	KT121519	
	10-May-13	Eurotiales	<i>Penicillium</i> sp.	3DF-1	KT121508	
	10-May-13	Capnodiales	<i>Cladosporium</i> sp.	3DF-2	KT121522	
	10-May-13	Eurotiales	<i>Penicillium rubens</i>	3DF-3	KT121507	
	10-May-13	Hypocreales	<i>Trichoderma harzianum</i>	3DF-4	KT121486	
	<i>Pycnoclavella communis</i>	4-Oct-12	Eurotiales	<i>Penicillium</i> sp.	PN-1b	KT121498
		4-Oct-12	Eurotiales	<i>Penicillium</i> sp.	PN-2b	KT121497
4-Oct-12		Hypocreales	<i>Bionectria</i> sp.	PN-3b	KT121530	
4-Oct-12		Eurotiales	<i>Talaromyces</i> sp.	PN-4b	KT121491	
4-Oct-12		Eurotiales	<i>Aspergillus fumigatus</i>	PN-5b	KT121531	
10-May-13		Hypocreales	<i>Trichoderma harzianum</i>	1PN-1	KT121490	
10-May-13		Hypocreales	<i>Trichoderma</i> sp.	1PN-2	KT121489	
10-May-13		Capnodiales	<i>Cladosporium</i> sp.	2PN-1	KT121526	
10-May-13		Capnodiales	<i>Cladosporium</i> sp.	2PN-2	KT121525	
10-May-13		Pleosporales	<i>Phoma</i> sp.	3PN-1	KT121496	
Seawater samples		10-May-13	Eurotiales	<i>Aspergillus</i> sp.	1SW-1	KT121534
	10-May-13	Eurotiales	<i>Penicillium rubens</i>	1SW-2	KT121513	
	10-May-13	Pleosporales	Pleosporales sp.	1SW-4	KT121493	
	10-May-13	Pleosporales	<i>Alternaria</i> sp.	1SW-5	KT121537	
	10-May-13	Eurotiales	<i>Aspergillus</i> sp.	1SW-6	KT121533	
	10-May-13	Hypocreales	<i>Trichoderma</i> sp.	2SW-1	KT121487	
	10-May-13	Pleosporales	<i>Alternaria</i> sp.	2SW-2	KT121536	
	10-May-13	Capnodiales	<i>Cladosporium</i> sp.	2SW-3	KT121524	
	10-May-13	Eurotiales	<i>Penicillium chrysogenum</i>	2SW-4	KT121523	
	10-May-13	Capnodiales	<i>Cladosporium</i> sp.	2SW-5	KT121503	
	10-May-13	Eurotiales	<i>Penicillium rubens</i>	3SW-1	KT121506	

**Table 1** (continued)

Ascidian species	Col. date	Fungal order	Potential species	Code	Acc. no.
	10-May-13	Capnodiales	<i>Cladosporium</i> sp.	3SW-2	KT121521
	10-May-13	Eurotiales	<i>Penicillium rubens</i>	3SW-3	KT121505
	10-May-13	Eurotiales	<i>Penicillium rubens</i>	3SW-4	KT121504
	10-May-13	Eurotiales	<i>Penicillium rubens</i>	3SW-5	KT121503

'Col. Date' indicates when the samples were collected (day, month and year). 'Code' corresponds to the unique code given to each isolate obtained in this study. 'Acc. No.' refers to the GenBank accession number of each isolate's ITS sequence

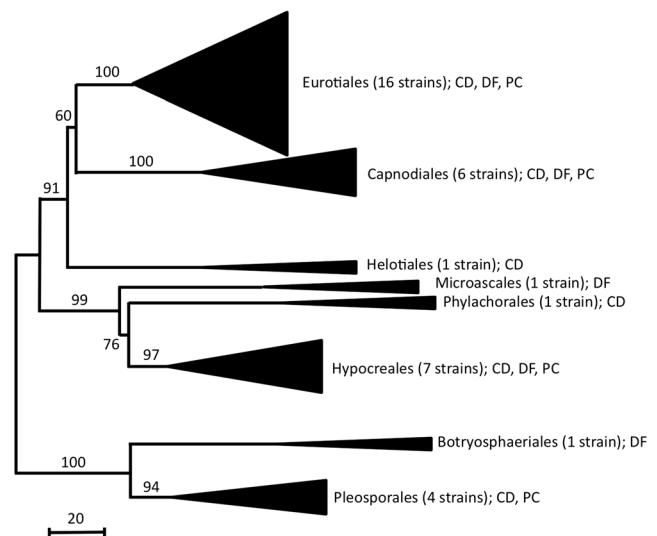
### 3 Results and discussion

A total of 52 different fungal strains were successfully isolated from all ascidian and seawater samples, except for *Pycnoclavella communis* collected on May 18, 2012 and all zooid dissections of *Cystodytes dellechiajei*. The absence of fungal isolates from *C. dellechiajei* zooids (where active filtration of seawater occurs) suggests that most fungi are located in the tunic of the animal and that the tunic represents a more suitable habitat for the survival or long-term storage of fungal spores. Moreover, fungi were observed growing out from the cut edges of the tunic (Fig. 1a), indicating that fungi are located within the tunic and do not just adhere to the surface exposed to the environment. Similarly, fungi isolated from *Didemnum fulgens* appeared to sprout from within the tunic (Fig. 1b), although in this case and also for *Pycnoclavella communis* (Fig. 1c), we were unable to separate zooids from their surrounding tunic and cannot determine exactly where fungal growth originated. Together, these observations suggest that at least some fungal propagules are not expelled or rapidly digested by the zooid, but rather reside (or are stored) in the tunic where they remain in a viable state for some time.

Of the 52 sequences obtained here, 37 sequences were retrieved from fungal isolates associated with ascidians and 15 from seawater samples. Ascidian-associated fungi were classified in eight distinct orders: Eurotiales, Capnodiales, Helotiales, Microascales, Phylachorales, Hypocreales, Botryosphaeriales and Pleosporales (Table 1). A high degree of host-specificity of ascidian-associated fungi was not apparent, since different individuals within the same host species appeared to contain different fungal isolates (Table 1). Differences among replicates could be due to a number of reasons, including different sampling times (and thus abiotic conditions) and intraspecific variability. Despite such variability, most of the fungal sequences retrieved for all host species and replicates were classified within the four fungal orders: Eurotiales, Pleosporales, Hypocreales, and Capnodiales (Table 1, Fig. 2). Moreover, all sequences obtained for *P. communis* and the seawater samples were classified within the above mentioned four orders (Table 1, Fig. 2). Similarly, Menezes et al. (2010) found that the most common fungi in the Brazilian ascidian *Didemnum* sp. (>10 % of all isolates)

belonged to three of the major orders reported here (Eurotiales, Pleosporales, and Hypocreales). Interestingly, these orders have also been commonly reported fungal orders in the Mediterranean sponge *Ircinia variabilis* (Paz et al. 2010) and in the Caribbean coral *Acropora formosa* (Yarden et al. 2007), and contain sequences of both marine and terrestrial origin.

Terrestrial fungal strains are often retrieved from marine invertebrates (e.g., Li and Wang 2009; Menezes et al. 2010; Paz et al. 2010) and, in most cases, are believed to represent spore contaminants, not true symbionts (reviewed in Suryanarayanan 2012). For instance, *Penicillium rubens* has been commonly retrieved from several macro-organisms, sand and seawater samples (Park et al. 2014) and was isolated from both *D. fulgens* and seawater samples herein. Similarly, *Aspergillus fumigatus* (order Eurotiales) is ubiquitous on land and has also been observed in association with diverse marine invertebrates (e.g., sea cucumbers, soft corals and *P. communis* herein). In contrast, some marine-derived fungi appear to exhibit host-specificity and be involved in host metabolism to



**Fig. 2** Neighbor-joining phylogenetic tree based on rRNA ITS sequences of cultured fungi associated with the Mediterranean ascidians *Cystodytes dellechiajei* (CD), *Didemnum fulgens* (DF), and *Pycnoclavella communis* (PC). Fungal order and the number of strains (in parenthesis) are also shown. Numbers above or below branches correspond to bootstrap values

some degree (e.g., producing or co-producing bioactive secondary metabolites). For example, *Epicoccum nigrum* (order Pleosporales) has been isolated from sponges, produces cytotoxic metabolites (Jiao et al. 2009; Sun et al. 2011), and here it was only observed in *C. dellechiajei*. These observations indicate some overlapping specificity patterns among fungi isolated from ascidians and other marine invertebrates, yet there remains little data regarding the host-specificity and ecological roles of ascidian-associated fungi.

Some fungal isolates obtained from the ascidians *C. dellechiajei* and *D. fulgens* belonged to non-commonly retrieved orders (Fig. 2) and matched to well-known plant pathogens. *C. dellechiajei* isolate 3CD-2 (Table 1) was classified within the order Helotiales and matched to *Botrytis cinerea* (100 % identity; 100 % coverage), a necrotrophic fungus responsible for gray mold on hundreds of dicot plants (Elad et al. 2007; Williamson et al. 2007). A second isolate from this ascidian species (1CD-1; Table 1) belonged to the order Phylachorales and matched to *Plectosphaerella cucumerina* (100 % identity; 100 % coverage), another well-known pathogen of several plant species (Carlucci et al. 2012) that has also been reported in marine sediments and that produces several bioactive secondary metabolites (Carr et al. 2009). Finally, isolate IDF-1 from *D. fulgens* was classified within the order Microascales and was closely related to *Scopulariopsis brevicaulis* (99 % identity; 100 % coverage), a human pathogen that can cause severe illnesses (e.g., skin lesions, pneumonia; Wheat et al. 1984; Dhar and Carey 1993; Cuenca-Estrella et al. 2003). More recently, *S. brevicaulis* strain LF580 was isolated from a Mediterranean sponge and was shown to produce scopularides A and B (Yu et al. 2008), both exhibiting specific activities against pancreatic and colon tumor cells (Imhoff et al. 2010). The role of some marine invertebrates as potential pathogens reservoirs has been previously suggested. For instance, the fungus *Aspergillus sydowii* can cause aspergillosis in Caribbean sea fans (Geiser et al. 1998; Alker et al. 2001) and a coral-infectious isolate has been isolated from an apparently healthy Bahamian sponge (Eingil et al. 2009). The finding of putative pathogenic fungi in ascidians indicates another possible reservoir of disease-causing propagules in marine invertebrates, although their relative importance remains largely unknown. Indeed, the transmission of a potential pathogen from its marine animal reservoir to its target has never been reported and it is not yet clear how a fungus may escape the inner tissues of an ascidian or a sponge and be released to the environment, to then become an active pathogenic agent. While the potential impact of zoonotic diseases on marine mammals has been drawing attention (including the threat posed by fungal pathogens; reviewed in Van Bressemer et al. 2009), and in spite of a growing awareness to marine invertebrate diseases (e.g., Webster 2007; Bourne et al. 2009) much less attention has been given to the potential zoonotic nature of some of these diseases.

Ascidians, like sponges, are active filter feeders and thus constantly filter huge volumes of seawater and remove microbes of all types from the water column. Depending on their size and nature, these microbes may be expelled from the ascidian, digested as prey items or remain undigested and accumulate in host tissues. Microbes present in the ascidian tunic or the sponge mesohyl may remain viable for a period of time, and in the case of bacteria, are able to reproduce and maintain stable communities for long periods of time (Erwin et al. 2012; López-Legentil et al. 2015). Fungi in the ascidian tunic are probably stored as spores, since fungal hyphae (easier to identify in light and electron micrographs than spores) have never been reported. The ecological role of these spores and the potential contribution of fungi to host metabolism and overall fitness remain unclear. However, evidence to date has shown a surprising diversity of fungi in ascidians (Menezes et al. 2010; this study), warranting further investigations into the significance and prevalence of this symbiotic relationship and for bioprospecting. In particular, future metagenomic and transcriptomic studies should provide a more comprehensive data set to assess which fungal taxa are establishing true symbiotic relationships with their host (if any) and their possible involvement in the production of bioactive secondary metabolites.

**Acknowledgments** This research was funded by the Spanish Government project MARSYMBIOMICS CTM2013-43287-P, and the Catalan Government grant 2014SGR-336 for Consolidated Research Groups.

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