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Peacock's tail with a fungal cocktail: first assessment of the mycobiota associated with the brown alga *Padina pavonica*

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ABSTRACT

The microbial diversity of the Mediterranean Sea is still poorly investigated, and a greater effort is needed to reveal marine fungal biodiversity associated with algal substrates. This study is the first description of the cultivable mycobiota associated with the calcareous brown alga *Padina pavonica*. Twenty algal thalli were analysed with a polyphasic approach, combining morphological and molecular data for fungal identification. Our data reveal a surprising richness of fungal species associated with a single brown alga: 268 isolates belonging to 134 taxa ascribable to Ascomycota (95.3%), Basidiomycota (5.2%), and Mucoromycota (0.7%) were retrieved. Twenty-nine species were reported for the first time from a marine environment. The analyses of the fungal community revealed possible substrate specificity. In addition, a number of sterile strains form separate clusters within the Diaporthales, Hypocreales, and Pleosporales, indicating that putative new lineages may arise from the marine environment.

1. Introduction

Biodiversity of fungi represents one of the most challenging and interesting fields in marine biology. Marine fungi can be defined as an ecologically diverse and cosmopolitan group of organisms distributed in all oceans and seas (Hyde et al., 1998), although the patterns and processes of their local and global diversity are yet to be disclosed. Functioning as saprobes, parasites and mutualistic symbionts (Jones, 2011; Raghukumar, 2017), marine fungi are heavily involved in biogeochemical cycling (Rédou et al., 2015). They produce numerous interesting compounds and enzymes, which, beside their ecological significance, have been acknowledged for their astonishing bioactivity (Mouad et al., 2011, 2012; Burgaud et al., 2014; Bonugli-Santos et al., 2015; Imhoff, 2016; Sridhar, 2017).

Jones et al. (2015) listed more than 1100 species of marine fungi, belonging to Ascomycota, Basidiomycota, Blastocladiomycota, and Chytridiomycota. Nevertheless, more than 10,000 species are estimated as yet to be discovered (Jones and Pang, 2012). This major gap is mainly due to the scarcity of habitats/substrates thoroughly explored for the presence of these organisms and to the low number of researchers currently working in this field.

In this context, the Mediterranean Sea, notwithstanding it is known as a hotspot of biodiversity for flora and fauna, is still a poorly investigated area (Coll et al., 2010). Only few data are available, in particular regarding algicolous fungi. Garzoli et al. (2014) presented the first characterisation of the fungal community associated with a red alga, *Asparagopsis taxiformis*, observing only 5 fungal taxa on 24 thalli; more recently, Gnavi et al. (2017), analysing the green alga *Flabellia petiolata,* detected 64 fungal taxa.

The present work is part of an extensive survey aimed to unravel the microbial diversity of the Mediterranean Sea by means of a polyphasic approach combining morphological, physiological, and molecular studies (Panno et al., 2013; Garzoli et al., 2014; 2015; Gnavi et al., 2014; Gnavi et al., 2017). The focus of the current report is on the culturable mycobiota associated with the brown alga *Padina pavonica* (Phaeophyceae, Dictyotales, Dictyotaceae), a widely distributed warm-temperate species (Guiry et al., 2014). Due to its fan-shaped thallus, *P. pavonica* is known also as Peacock's tail and it is commonly found in the Mediterranean Sea (Cormaci et al., 2012). This alga is rich in carbohydrates (mainly alginates and laminarans), lipids (fucosterol), vitamins, and mineral salts, and it is known for its slight bioactivity against microbial pathogens (Kamenarska et al., 2002; Awad et al., 2008; Omezzine et al., 2009). Although previous attempts have been made to study its mycobiota

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(Cuomo et al., 1983; Abdel-Gawad et al., 2014), the published data were exclusively based on morphological observations and did not provide a complete list of fungal species associated with this alga. To the best of our knowledge, this study represents the first description of the culturable mycobiota associated with *P. pavonica*.

2. Materials and methods

2.1. Sampling

Samples of *P. pavonica* were collected in March 2010 in the coastal waters of Elba Island (Livorno, Italy) in the Tyrrhenian Sea (NW Mediterranean Sea). The same two sampling sites considered by Gnavi et al. (2017) for the presence of fungi associated with the green alga *F. petiolata* were chosen in the seagrass meadows of *Posidonia oceanica*: (1) Ghiaie (WGS84 42°49′04″N, 10°19′20″E) and (2) Margidore (WGS84 42°45′29″N, 10°18′24″E). Twenty thalli (10 thalli from each sampling site) were harvested at two different depths (5 thalli/each depth): USL, Upper Sea Level (average depth: 3–5 m bsl); ISL, Intermediate Sea Level (average depth: 14–15 m bsl). Algae were maintained in sterile dark containers at 4 °C.

2.2. Fungal isolation

Algal thalli were sonicated (30 s each time) and serially washed in artificial sterilized SeaWater (SW, 3.4% w/v Sea Salt mix - Sigma-Aldrich, Saint Louis, USA - in ultrapure distilled water ddH_2O) three times to remove unrefined sediments. According to the procedure described by Gnavi et al. (2017), each thallus was homogenized in 20 mL of filtered seawater by means of Ultra-Turrax (IKA) sterile device. The homogenates were further diluted 1:10 in sterilized seawater and 1 mL of the resulting solution was plated in sterile Petri dishes (120 mm) containing 30 mL of one of two media: 1) Corn Meal Agar SeaWater medium (CMASW, 17 g CMA - Sigma-Aldrich Saint Louis, USA - dissolved in 1 L of filtered SW) and 2) *Padina* Agar SeaWater medium (PASW, 1 g fw of *P. pavonica* in 100 mL of SW boiled for 30 min at 60 °C and filtered; 18 g agar; SW up 1 L) (Panno, 2014). Both media were supplemented with antibiotics (Gentamicin 80 mg/L, Tazocin 100 mg/L). Plates were prepared in triplicate, incubated at 15 °C for 15 d and subsequently placed at 24 °C for 45 d to allow the isolation of both psychrotolerant and mesophilic colonies. Colony forming units per gram of dry weight for each thallus (CFU/g dw) were recorded and strains from each fungal morphotype and from each sampling site were isolated in pure culture and preserved at the *Mycotheca Universitatis Taurinensis* (MUT). Accession records and descriptions are available at MUT website (www.mut.unito.it/en; Supp. Mat. 1).

2.3. Fungal identification

Strains were firstly identified on the basis of their macro/microscopic morphological and physiological features according to specific taxonomical keys as described in Ainsworth (2008). Subsequently, specific markers were amplified in a Biometra TGradient Thermocycler (Biometra, Göttingen, Germany): the nuclear ribosomal nrDNA partial regions (ITS or LSU and SSU when necessary) were amplified using the universal primers ITS1/ITS4 (White et al., 1990; Schoch et al., 2012), LR0R/LR7 (Lapeyre et al., 1993), and NS1/NS4 (White et al., 1990). For yeasts, NL1/NL4 primers were used to amplify the D1/D2 region of LSU (Kurtzman and Robnett, 1997). For strains belonging to *Aspergillus* spp. and *Penicillium* spp., the β-tubulin gene was amplified using the primer pair Bt2a/Bt2b (Glass and Donaldson, 1995); ACT512F/ACT783R (Carbone and Kohn, 1999) were used to amplify the actin gene for *Cladosporium* spp. Finally, for *Stachybotrys* and *Alternaria* spp., it was necessary to amplify respectively calmodulin and glyceraldehyde-3-phosphate dehydrogenase genes, using primers CL-1/CL-2 (O'Donnell et al., 2000) and GPD1/GPD2 (Berbee et al., 1999). PCR products were then sequenced using Sanger sequencing techniques at Macrogen (Seoul Korea). The resulting electropherograms (ABI-files) were analysed with Sequencer software, version 5.2 (Gene Codes Corp., Ann Arbor, MI, USA). Newly generated sequences were compared to those available in public databases (GenBank - nblast; mismatch 1/-2; gap costs linear; Mycobank) and deposited at NCBI (Suppl. Mat. 1). Sterile mycelia and strains with morphological features that did not match any available species description and showed low sequence similarity with those available in public databases were further characterised through phylogenetic inference.

2.4. Phylogenetic analysis

A full phylogenetic analysis was performed on ITS sequences. Three datasets were composed for Diaporthales, Hypocreales and Pleosporales, following the *Outline of Ascomycota* (Lumbsch and Huhndorf, 2011). Sequences from the Fungal Biodiversity Center CBS type strains were preferred, when available; other sequences were chosen on the basis of specific taxonomic studies and retrieved from NCBI GenBank database. Alignments were performed with MUSCLE, implemented in MEGA 7.0, using default conditions for gap openings and gap extension penalties and trimmed by TrimAl (v 1.2) (http://trimal.cgenomics.org) with the AUTOMATED1 setting. Phylogenetic analyses were performed

Table 1
Average fungal load (CFU/g dw±SE) and number of fungal entities isolated from *P. pavonica* specimens in different sites and depth. Biodiversity within sampling sites: Shannon-Weaver index (H′), Gini-Simpson index (1-Lambda) and Pielou's evenness (J′).

Sites	Ghiaie				Margidore			
Depth	USL 3-5m depth		ISL 14-15m depth		USL 3-5m depth		ISL 14-15m depth	
media	PASW	CMASW	PASW	CMASW	PASW	CMASW	PASW	CMASW
CFU/g dw±SE	1,485±52	1,007±94	1,760±56	1,500±79	1,523±45	1,083±71	1,343±90	898±71
Average CFU/gdw	1,246±41		1,630±51		1,303±37		1,121±41	
	1,438±69				1,212±72			
Num. of exclusive fungal taxa (total number of taxa retrieved)	15(21)	8(17)	21(42)	13(30)	13(31)	7(22)	13(24)	4(15)
	23(35)		37(64)		23(44)		17(33)	
	65(90)				44(69)			
	Diversity indices							
H'	3.203		3.413		3.512		3.226	
1-lambda	0.938		0.920		0.962		0.947	
J'	0.901		0.821		0.928		0.923	