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International Tailored Chemotherapy Adjuvant (ITACA) Trial, a Phase III Multicenter Randomized Trial Comparing Adjuvant Pharmacogenomic-Driven Chemotherapy versus Standard Adjuvant Chemotherapy in completely Resected Stage II-IIIA Non-Small Cell Lung Cancer

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Sink or swim: Updated knowledge on marine fungi associated with wood substrates in the Mediterranean Sea and hints about their potential to remediate hydrocarbons

- [Laura Garzoli^{a, b, 1, 2,}](#)
- [Giorgio Gnani^{b, 2,}](#)
- [Federica Tamma^{a, 1,}](#)
- [Solveig Tosi^{a, 1,}](#)
- [Giovanna C. Varese^{b, 2,}](#)
- [Anna M. Picco^{a,}](#)

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Highlights

- The paper provides an updated checklist of Mediterranean lignicolous marine fungi.
- The majority of wood samples were colonized by fungi and 40 taxa were identified.
- The isolated strains were shown to be able to grow on Crude Oil and/or BTZ.
- Their performances were enhanced in the presence of NaCl.
- The species isolated are now available in MUT Culture Collection.

Abstract

This paper provides the first update in more than twenty years on the available knowledge about lignicolous marine fungi in the Mediterranean Sea. Fungi found on collected wood samples were analyzed using a combination of morphological and molecular techniques. Almost 90% of the samples were colonized by fungi. The total number of recorded taxa, which amounted to 57 in the late 1990s, has now risen to 93. Wood-inhabiting marine fungi are good producers of ligninolytic enzymes, which can degrade several aromatic and recalcitrant environmental pollutants. In light of bioremediation technologies, this study also evaluated the potential of the isolated strains to remediate complex hydrocarbon substrates. Seventeen isolates were shown to be able to grow on hydrocarbon media as a sole carbon source; enhanced performances were achieved in the presence of NaCl, suggesting that these fungi adapt well to marine conditions and confirming that salt can trigger specific metabolic pathways in marine fungi.

1. Introduction

Fungi have been found to be the main decomposers of woody (and herbaceous) substrates that enter marine ecosystems. They are the principal agents of enzymatic lignocellulose degradation, particularly in the upper-intertidal region where marine borers are unable to survive ([Hyde et al., 1998](#) and [Panno et al., 2013](#)).

A delineation of marine fungi is still controversial. Marine fungi were first defined on the basis of their physiological features, such as the ability to grow at certain seawater concentration ([Johnson and Sparrow, 1961](#) and [Tubaki, 1969](#)); however, the traditionally accepted and most used definition was given by [Kohlmeyer and Kohlmeyer \(1979\)](#): “*obligate*” marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitats; *facultative marine fungi* are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in marine environment”. According to this definition, 537 species are currently considered “obligate” marine species ([Jones, 2011](#)). Although this definition is still used, in recent years authors tried to enlarge this concept, in order to overcome the issue related to the proliferating number of so-called “marine-derived” strains (i.e. “terrestrial” strains highly adapted to marine environment). The ability to germinate and to form mycelium under natural marine conditions is considered as a valid criterion for classifying an organism as a marine fungus ([Kohlmeyer and Volkmann-Kohlmeyer, 1991](#)). Nowadays, marine fungi are an ecological rather than a taxonomical group, which comprises organisms belonging to different *taxa* that share eco-physiological features ([Hyde et al., 1998](#)). Following this new definitions, [Jones \(2011\)](#) highlighted a wide range of fungi that should be considered as marine, and estimates that their biodiversity may exceed 10,000 *taxa*.

Although there are many studies available on marine fungal communities associated with wood substrates, the last report on the Mediterranean Sea dates back to 1990 ([Jones, 2010](#)). The main problem with this lack of update is that up to 1998 it was common to only record obligate marine species because the others, which are now recognized as being facultative marine species, were regarded as being mere terrestrial contaminants without ecological roles ([Hyde et al., 1998](#)). Therefore, in all the available studies ([Kohlmeyer, 1963](#), [Jones et al., 1972](#), [Montemartini, 1979](#), [Furtado and Jones, 1980](#), [Grasso et al., 1985](#), [Grasso et al., 1990](#) and [Cuomo et al., 1988](#), Fig. 1), the authors did not report (or rarely reported) species that were considered to be of terrestrial origin (e.g. *Fusarium*, *Trichoderma* and *Verticillium* spp.). In addition, these mycoflorae were never investigated by means of molecular phylogenetic tools.



Fig. 1.

Sampled sites in current and past studies in the Mediterranean Sea.

The introduction of molecular identification has shown that fungal biodiversity is considerably higher than it was thought to be, especially in marine environments. For example, [Cuomo et al. \(1988\)](#) only recovered 41 fungi on driftwood (5373 samples) and 34 on submerged timber test blocks (1089 samples) from 13 locations around the Italian coastline. Other studies also reported low diversity, despite the large number of substrates investigated. On the basis of these considerations, the main aim of the present study was to provide an update of the available data on lignicolous marine fungi associated with naturally occurring wood substrates along the coast of the Italian peninsula, in light of modern molecular tools.

In the marine environments fungi can live in extreme conditions and have shown to be active in particular in the biodegradation of organic matter, chitin (mainly exoskeletons of crustacea) and lignin (drift- or submerged wood). Lignicolous species have been found on driftwood, pilings, wooden boats, mangrove roots and trunks ([Shearer et al., 2007](#)), from the surface to a depth below 1600 m ([Dupont et al., 2009](#)); the majority of them are Ascomycota, mainly belonging to Halosphaeriaceae (Microascales, Sordariomycetes) and Lulworthiales (Sordariomycetes) ([Spatafora et al., 1998](#)). Wood-inhabited marine fungi can produce extracellular enzymes such as peroxidases and laccases that are capable of degrading or modifying not only lignin but also several aromatic and recalcitrant environmental pollutants. Such pollutants can be found in Crude Oil waste ([Damare et al., 2012](#)), gallons of which are spilled on a daily basis from chronic sources worldwide ([Fingas, 2010](#)). Oil spills at sea are considered to be one of the most serious environmental issues; thus, besides taking effective measures to prevent spills in marine and terrestrial environments, there is

an increasing need for cost-effective remediation technologies ([Harms et al., 2011](#)). Marine fungi from woody substrates appear as suitable candidates for such technologies both because they are an integral part of the coastal communities and because they tolerate saline conditions ([Passarini et al., 2011](#)). Therefore, the second aim of this study was to investigate the bioremediation potential of lignicolous marine strains towards complex hydrocarbon substrates.

2. Materials and methods

2.1. Fungal isolation and identification

Wood samples were collected during summer 2011 in different sites along the Italian coastline: the Marine Protected Area (MPA) “Island of Bergeggi”, the MPA “Portofino”, Zoagli, the MPA “Cinque Terre”, and Porto Badisco ([Fig. 1](#)).

According to the definitions proposed by [Hughes \(1968\)](#), in the present study, driftwood referred to wood that was floating or found loose on the shore, and submerged wood referred to wood that was found on the bottom, constantly submerged by seawater. In both cases, samples were only considered for analysis if they showed visible signs that they had been in contact with seawater for at least one year (e.g. bore holes made by gribbles and shipworms). Wood samples were collected in sterile containers and were transported at 4 °C. They were processed in the laboratory according to the following modified version of the procedure described by [Vrijmoed \(2000\)](#): (1) the wood samples were scraped and washed under running tap water to remove the sediment layer, debris and other surface fouling organisms; (2) samples were then washed serially (three times) in autoclaved seawater in sterile conditions under laminar flow; (3) fragments measuring about 1 cm³ were subsequently cut by means of sterile shears; (4) three fragments per sample were placed into three sterile 90 mm Petri dishes (three in each) and covered with autoclaved seawater supplemented with an antibiotic mixture (70 mg l⁻¹ Penicillin, 200 mg l⁻¹ Streptomycin, 50 mg l⁻¹ Chloramphenicol) to prevent bacterial growth; (5) the three Petri dishes for each sample were incubated at three different temperatures: 4 °C, 15 °C, 25 °C. Three replicas were set up for each wood sample at each temperature. The plates were checked weekly for the first month, and then monthly for six months.

Strains from each fungal morphotype from each sampling site were isolated in pure culture and deposited in the Culture Collections of Pavia (PAV-M accession numbers from 1.002 to 1.171D) and Turin (MUT accession numbers comprises between 1483 and 3425).

Taxonomic identification of the isolated strains was carried out using morphological and molecular approaches. Firstly, fungi were identified morphologically on the basis of specific taxonomical keys. Subsequently, molecular analyses were performed by sequencing the Internal Transcribed Spacer region ITS rDNA ([White et al., 1990](#)), the partial large ribosomal subunit LSU rDNA ([Vilgalys and Hester, 1990](#)), and the actin gene (*ACT*) (for *Cladosporium* spp., [Carbone and Kohn, 1999](#)). Taxonomic assignments were based on similarity to reference sequences of the GenBank (nblast; mismatch 1/-2; gap costs linear) and CBS databases ([Supp. mat. 1](#)). Data related to the microfungi isolated in this study were deposited in the NCBI database (Accession numbers KF915979-KF916005, KF937822-KF937824, KM203360-KM203370, KM264275-KM264278). Strains were checked morphologically to confirm the identifications on the bases of Blasts results.

2.2. Growth trials on Crude Oil and BTZ

Twenty isolates were selected (all the obligate marine strains and 15 strains randomly chosen between the isolates) and tested on two different hydrocarbon substrates: Crude Oil (CO), from the oil well platform “Rospo di Mare” (Adriatic Sea) and BTZ, a heavy fuel oil with sulfur content (<1% in weight). Hydrocarbons (150 µl) were supplied on 60 mm sterile plates on agarized water media: WA (Water Agar: 15 g Agar in 1000 ml dH₂O) and WA + NaCl (3.4% – average salt concentration of seawater).

The selected fungal strains were grown for 15 days on PDA (Potato Dextrose Agar: 4 g Potato extract, 20 g glucose, 15 g Agar dissolved in 1000 ml dH₂O) at 25 °C. Fifty microliters of a conidial suspension (10³ conidia ml⁻¹) or grounded mycelium (in case of sterile strains) was inoculated on the agar plates containing the hydrocarbons. Three replicas were set up for each fungus and growing substrates (CO, CO + NaCl, BTZ, BTZ + NaCl). Plates were incubated at RT and checked weekly up to three weeks. The colony diameter (and the presence of a clarification halo) was recorded ([Fig. 2](#)). Negative controls, consisting of non-inoculated plates and of inoculated plates without the hydrocarbons (with and without NaCl), were also incubated and checked.

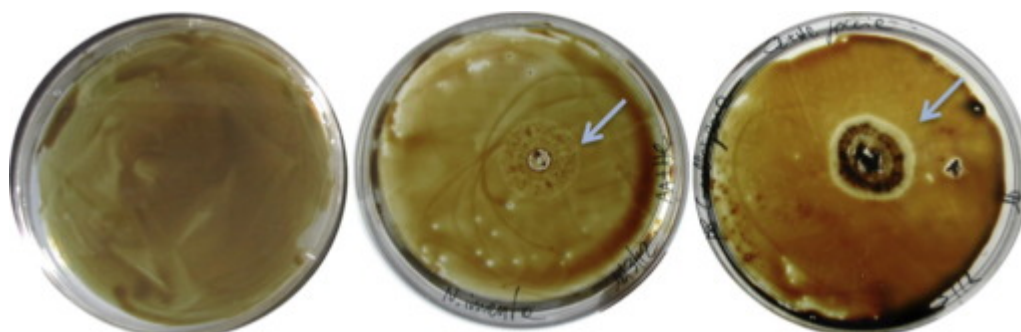


Fig. 2.

Growth of marine strains on hydrocarbon substrates: from left to right, no growth (–), growth without a clarification halo (+), growth with production of a clarification halo (+*).

[Figure options](#)

3. Results

3.1. Characterization of the mycoflora associated with wood substrates

88% of the analyzed wood samples (25) were colonized by fungi. A total of 40 taxa were identified ([Table 1](#)), of which 19 were isolated exclusively on driftwood, 12 on submerged wood, and 9 on both substrates.

Table 1.

Marine fungal species recovered on wood substrates along the Italian peninsula.

Taxa	% of occurrence		References		
	SW ^a (n.13)	DW ^a (n.12)	From WS ^a	From Med ^a	From ME ^a
ASCOMYCOTA					
<i>Acremonium implicatum</i> (J.C. Gilman & E.V. Abbott) W. Gams	0	8	FR ^a	[1], [2]	[3], [4], [5]
<i>Acremonium tubakii</i> W. Gams	8	0	FR ^a	[1]	[6]
<i>Acremonium furcatum</i> Moreau & F. Moreau ex Gams	8	0	FR ^a	FR ^a	[39]
<i>Acremonium</i> sp.	8	8			
<i>Alternaria tenuissima</i> (Kunze) Wiltshire	17	8	[7]	[8]	[3], [9], [10]
<i>Aspergillus ochraceus</i> G. Wilh.	8	8	FR ^a	[11], [12], [19]	[10], [12], [14]
<i>Aspergillus protuberus</i> Munt.-Cvetk.	8	0	FR ^a	[12]	[17], [18]
<i>Botryotrichum piluliferum</i> Sacc. & Marchal	0	8	FR ^a	[12], [19]	[19]
<i>Cadophora luteo-olivacea</i> (J.F.H. Beyma) T.C. Harr. & McNew	15	0	[7]	FR ^a	[5], [20]
<i>Capronia kleinmondensis</i> Marinc., M.J. Wingf. & Crous	8	0	FR ^a	FR ^a	FR ^a
<i>Ceriosporopsis halima</i> Linder	0	8	[21]	[21]	[7], [22]
<i>Cladosporium halotolerans</i> Zalar, de Hoog & Gunde-Cim.	8	0	FR ^a	[11]	[5], [8], [23]
<i>Clonostachys rosea</i> f. <i>rosea</i> (Link) Schroers, Samuels, Seifert & W. Gams	8	8	FR ^a	[1]	[24]
<i>Corollospora maritima</i> Werdem	23	42	[21]	[21]	[22]
<i>Engyodontium album</i> (Limber) de Hoog	8	8	FR ^a	[13]	[4]
<i>Exophiala angulosporea</i> Iwatsu, Udagawa & T. Takase	0	8	FR ^a	FR ^a	[25] [26]
<i>Fusarium oxysporum</i> Schltdl.	0	8	[19]	[12], [19]	[19], [28], [31]
<i>Gibberella avenacea</i> R.J. Cook	8	0	FR ^a	FR ^a	[31]
<i>Gibberella fujikuroi</i> (Sawada) Wollenw.	8	0	FR ^a	FR ^a	[27], [28]
<i>Gibberella stilboides</i> W.L. Gordon ex C. Booth	0	17	FR ^a	FR ^a	FR ^a
<i>Gibellulopsis nigrescens</i> (Pethybr.) Zare, W. Gams & Summerb.	8	0	FR ^a	[1]	[1]
<i>Haematonectria haematococca</i> (Berk. & Broome) Samuels & Rossman	31	8	[29]	[8], [12], [19]	[30], [31]

Taxa	% of occurrence		References		
	SW ^a (n.13)	DW ^a (n.12)	From WS ^a	From Med ^a	From ME ^a
<i>Ilyonectria radiculicola</i> (Gerlach & L. Nilsson) P. Chaverri & C. Salgado	8	0	FR ^a	FR ^a	[28]
<i>Lulwoana</i> sp. ^b	23	33			
<i>Niesslia exilis</i> (Alb. & Schwein.) G. Winter	0	8	[7]	FR ^a	[7]
<i>Penicillium expansum</i> Link	23	0	FR ^a	[1]	[31]
<i>Penicillium citrinum</i> Thom	0	8	[32]	[8]	[18]
<i>Penicillium</i> sp.	0	17			
<i>Pseudallescheria boydii</i> (Shear) McGinnis, A.A. Padhye & Ajello	0	8	FR ^a	FR ^a	[33]
<i>Purpureocillium lilacinum</i> (Thom) Luangsa- ard, Houbraken, Hywel-Jones & Samson	0	8	FR ^a	[12]	[34], [35], [36]
<i>Scolecobasidium arenarium</i> (Nicot) M.B. Ellis	0	8	[37]	[37]	[37]
<i>Scopulariopsis hibernica</i> A. Mangan	0	8	FR ^a	FR ^a	FR ^a
Sordariomycetes sp.1	0	8			
Sordariomycetes sp.2	0	17			
Sordariomycetes sp.3	0	17			
<i>Trichoderma atroviride</i> P. Karst.	8	0	FR ^a	[8], [38]	[38]
<i>Trichoderma</i> sp.	0	8			
ZYGOMYCOTA					
<i>Rhizopus stolonifer</i> (Ehrenb.) Vuill.	0	8	FR ^a	[19]	[28]
Uncultured Coelomycetes	0	8			
Uncultured mycelia sterilia ^b	8	17			
Number of different taxa	21	28			

References: [1] [Panno et al. \(2013\)](#), [2] [Paz et al. \(2010\)](#), [3] [Zhang et al. \(2013\)](#), [4] [Oren and Gunde-Cimerman \(2012\)](#), [5] [Bubnova et al. \(2014\)](#), [6] [Zuccaro et al. \(2008\)](#), [7] [Rämä et al. \(2014\)](#), [8] [Sepcic et al. \(2011\)](#), [9] [Sun et al. \(2013\)](#), [10] [Borzykh and Zvereva \(2012\)](#), [11] [Zajc et al. \(2012\)](#), [12] [Muntañola-Cvetković and Ristanović \(1980\)](#), [13] [Proksch et al. \(2010\)](#), [14] [Cui et al. \(2009\)](#), [17] [Mathan et al. \(2011\)](#), [18] [Furbino et al. \(2014\)](#), [19] [Abdel-Azeem and Salem Fatma \(2012\)](#), [20] [Overy et al. \(2014\)](#), [21] see [Table 2](#), [22] [Jones et al. \(2009\)](#), [23] [Bensch et al. \(2012\)](#), [24] [Clipson et al. \(2001\)](#), [25] [Gjessing et al. \(2011\)](#), [26] [Nyaoke et al. \(2009\)](#), [27] [Rhoobunjongde et al. \(1991\)](#), [28] [Khallil et al. \(1991\)](#), [29] [Fryar et al. \(2004\)](#), [30] [Sarmiento-Ramírez et al. \(2010\)](#), [31] [Pindi \(2012\)](#), [32] [Ravikumar et al. \(2009\)](#), [33] [Phillott et al. \(2004\)](#), [34] [Raghukumar and](#)

	<u>Kohlmeyer (1963)</u>	<u>Jones et al. (1972)</u>	<u>Montemartini (1979)</u>	<u>Furtado and Jones (1980)</u>	<u>Grass et al. (1985)</u>	<u>Cuomo et al. (1988)</u>	<u>Grass et al. (1990)</u>	Current study
<i>Aspergillus protuberus</i>	—	—	—	—	—	—	—	X
<i>Asteromyces cruciatus</i>	—	—	—	—	—	X	—	—
<i>Botryotrichum piluliferum</i>	—	—	—	—	—	—	—	X
<i>Cadophora luteo-olivacea</i>	—	—	—	—	—	—	—	X
<i>Capronia kleinmondensis</i>	—	—	—	—	—	—	—	X
<i>Cephalotrichum stemonitis</i> (as <i>Doratomyces stemonitis</i>)	—	—	X	—	—	—	—	—
<i>Ceriosporopsis calyptrata</i>	X	—	—	—	—	—	—	—
<i>Ceriosporopsis circumvestita</i>	—	—	—	—	X	X	—	—
<i>Ceriosporopsis halima</i>	X	—	X	X	X	X	X	X
<i>Chaetosphaeria chaetosa</i>	—	—	—	—	—	X	—	—
<i>Cirrenalia macrocephala</i>	X	X	X	—	X	X	X	—
<i>Cirrenalia pseudomacrocephala</i>	—	—	—	X	—	X	—	—
<i>Cladosporium halotolerans</i>	—	—	—	—	—	—	—	X
<i>Cladosporium</i> sp.	—	—	—	X	—	—	—	—
<i>Clonostachys rosea</i> f. <i>rosea</i>	—	—	—	—	—	—	—	X
<i>Corollospora cristata</i> (as <i>Nereiospora cristata</i>)	—	—	—	—	—	X	—	—
<i>Corollospora maritima</i>	X	X	X	X	X	X	X	X
<i>Corollospora trifurcata</i> (as <i>Arenariomyces trifurcatus</i>)	—	—	—	—	X	X	X	—

	<u>Kohlmeyer (1963)</u>	<u>Jones et al. (1972)</u>	<u>Montemartini (1979)</u>	<u>Furtado and Jones (1980)</u>	<u>Grass et al. (1985)</u>	<u>Cuomo et al. (1988)</u>	<u>Grass et al. (1990)</u>	Current study
<i>Kirschsteiniothelia maritima</i>	—	—	—	—	—	X	—	—
<i>Leptosphaeria marina</i>	—	—	—	—	—	X	—	—
<i>Leptosphaeria orae-maris</i>	X	—	—	—	—	X	—	—
<i>Lignincola laevis</i>	—	—	—	X	X	X	X	—
<i>Lindra thalassiae</i> -like	—	—	—	—	X	—	—	—
<i>Lulwoana uniseptata</i>	X	X	X	X	X	X	X	—
<i>Lulwoana</i> sp.	—	—	—	—	—	—	—	X
<i>Lulworthia floridana</i>	—	—	—	X	X	—	—	—
<i>Lulworthia grandispora</i>	—	—	—	X	—	—	—	—
<i>Lulworthia kniepii</i> -like	X	—	—	—	—	X	—	—
<i>Lulworthia purpurea</i>	—	—	—	X	—	X	—	—
<i>Lulworthia</i> sp.	X	—	X	—	—	X	—	—
<i>Naïs inornata</i>	—	—	—	—	—	X	—	—
<i>Nautosphaeria cristaminuta</i>	—	—	—	—	—	X	—	—
<i>Nereiospora comata</i>	—	—	—	—	X	—	—	—
<i>Niesslia exilis</i>	—	—	—	—	—	—	—	X
<i>Papulaspora halima</i>	—	—	X	—	X	X	—	—
<i>Paraliomyces lentifer</i> (as <i>Paraliomyces lentiferus</i>)	—	—	—	—	—	X	—	—
<i>Penicillium expansum</i>	—	—	—	—	—	—	—	X
<i>Penicillium citrinum</i>	—	—	—	—	—	—	—	X
<i>Penicillium</i> sp.	—	—	—	—	—	—	—	X
<i>Periconia prolifica</i>	—	—	—	—	—	X	—	—
<i>Phoma</i> sp.	—	—	—	X	—	X	—	—
<i>Piricauda pelagica</i> (as	X	—	—	X	X	X	X	—

	Kohlmeyer (1963)	Jones et al. (1972)	Montemartini (1979)	Furtado and Jones (1980)	Grass et al. (1985)	Cuomo et al. (1988)	Grass et al. (1990)	Current study
<i>Trichoderma</i> sp.	—	—	—	—	—	—	—	x
<i>Zalerion varia</i> (as <i>Zalerion varium</i>)	—	—	—	x	—	—	—	—
BASIDIOMYCOTA								
<i>Nia vibrissa</i>	—	—	x	—	x	x	x	—
ZYGOMYCOTA								
<i>Rhizopus stolonifer</i>	—	—	—	—	—	—	—	x
Unknown Coelomycetes	—	—	—	—	—	—	—	x
Number of different taxa	12	9	14	19	20	43	16	39

Almost all the recorded taxa belonged to Ascomycota (36). Only one Mucoromycotina was found: *Rhizopus stolonifer*. The taxa that were most frequently isolated from driftwood were *Corollospora maritima* (42% of the samples), strains of *Lulwoana* sp. (33%), *Gibberella stilboides* and strains of *Penicillium* spp. (17%), strains of Sordariomycetes (sp.1 17% and sp.2 17%), and uncultured sterile mycelia (17%). The taxa that were most frequently isolated from submerged wood were *Haematonectria haematococca* (31% of the samples), *C. maritima*, *Lulwoana* sp. and *Penicillium expansum* (23%), *Alternaria tenuissima* (17%), *Cadophora luteo-olivacea* (15%). The frequency of the remaining taxa was found to be lower than 10%.

Melanised uncultured sterile mycelia were recorded on 12% of the analyzed samples (8% and 17% of submerged wood and driftwood, respectively), namely those that specifically did not show any *in vivo* sporulation and that were not capable of growing *in vitro*, despite many attempts to find suitable media.

3.2. Screening for the potential to remediate hydrocarbons

Results of growth trials on CO and BTZ are summarized in [Table 3](#). Seventeen out of the 20 analyzed strains displayed the ability to grow on hydrocarbons as a sole carbon source and the majority showed an increased growth rate in the presence of salt. All the fungi displayed null or explorative growth on the control plates without hydrocarbons. More specifically, 12 strains were capable of growing on both substrates, 2 were only capable of growing on BTZ, and 3 were only capable of growing on CO. The highest growths (in the presence and absence of NaCl) were displayed on both substrates by *A. tenuissima*, *G. stilboides* and *Niesslia exilis*, on CO by *C. maritima* strain 2 and *Penicillium citrinum*, and on BTZ by *Capronia kleinmondensis*, *Gibellulopsis nigrescens*, *H. haematococca*, *Ilyonectria radiculicola*, *Penicillium* sp.1, *Pseudoallescheria boydii* and *Purpureocillium lilacinum*. It is interesting to note that *C. kleinmondensis*, *G. nigrescens* and *Penicillium* sp.1 produced a clarification halo on the BTZ medium, and only in the presence of

NaCl in the last two cases. Different strains of *C. maritima* were tested and showed different abilities to use hydrocarbons.

Table 3.

Growth trials of marine isolates on BTZ and Crude Oil.

Taxa	BTZ		Crude Oil	
	WA	WA + NaCl	WA	WA + NaCl
<i>Acremonium implicatum</i>	–	–	–	–
<i>Alternaria tenuissima</i>	50	50	55	55
<i>Capronia kleinsondensis</i>	40*	67*	–	–
<i>Ceriosporopsis halima</i>	25	25	–	3
<i>Corollospora maritima</i> strain 1	–	–	5	7
<i>Corollospora maritima</i> strain 2	9	34	–	12
<i>Corollospora maritima</i> strain 3	24	20	55	4
<i>Exophiala angulospora</i>	–	–	2	9
<i>Gibberella stilboides</i>	50	50	55	55
<i>Gibellulopsis nigrescens</i>	55	55*	35	35
<i>Haematonectria haematococca</i>	51	43	35	18
<i>Ilyonectria radicola</i>	55	55	–	–
<i>Lulwoana</i> sp.	–	–	–	–
<i>Niesslia exilis</i>	45	55	55	55
<i>Penicillium citrinum</i>	30	33	50	43
<i>Penicillium</i> sp.1	32	47*	35	42
<i>Pseudoallescheria boydii</i>	51	48	26	30
<i>Purpureocillium lilacinum</i>	46	55	45	40
<i>Scolecobasidium arenarium</i>	–	–	–	–
<i>Sordariomycetes</i> sp.1	–	–	–	11

0.1–24 mm average diameter.

25–49 mm average diameter.

≥ 50 mm average diameter.

*Clarification halo.

(Standard deviation was lower than 0.5).

[Table options](#)

4. Discussion

Although wood in marine environment is one of the most commonly studied substrates for the presence of marine fungi, updated information for the Mediterranean Sea is still scarce ([Jones, 2010](#)). This study provides the first update for more than twenty years on the available knowledge about Mediterranean lignicolous marine mycoflora. In the present study, as in previous research, Ascomycota was found to be the most common taxon, thus confirming that this phylum dominates marine environments ([Jones et al., 2009](#) and [Jones and Pang, 2012](#)). The most frequent species found in our isolates were obligate marine species such as *C. maritima* ([Fig. 3a](#)) and *Lulwoana* sp. These results are in line with previous studies on wood substrates in the Mediterranean Sea ([Jones, 2010](#)). The genus *Lulwoana* belongs to the Lulworthiales order that comprises only marine ascomycetes ([Kohlmeyer et al., 2000](#)). Although this genus encompasses only one species, *L. uniseptata* (anamorph: *Zalerion maritimum*), neither morphological (isolates in pure culture did not produce propagules) nor molecular analyses (low homology – <82% – with the NCBI available sequences of *L. uniseptata* and high homology – >99% – with sequences indicated as *Lulwoana* sp.) allow us to assign our isolates to this species.

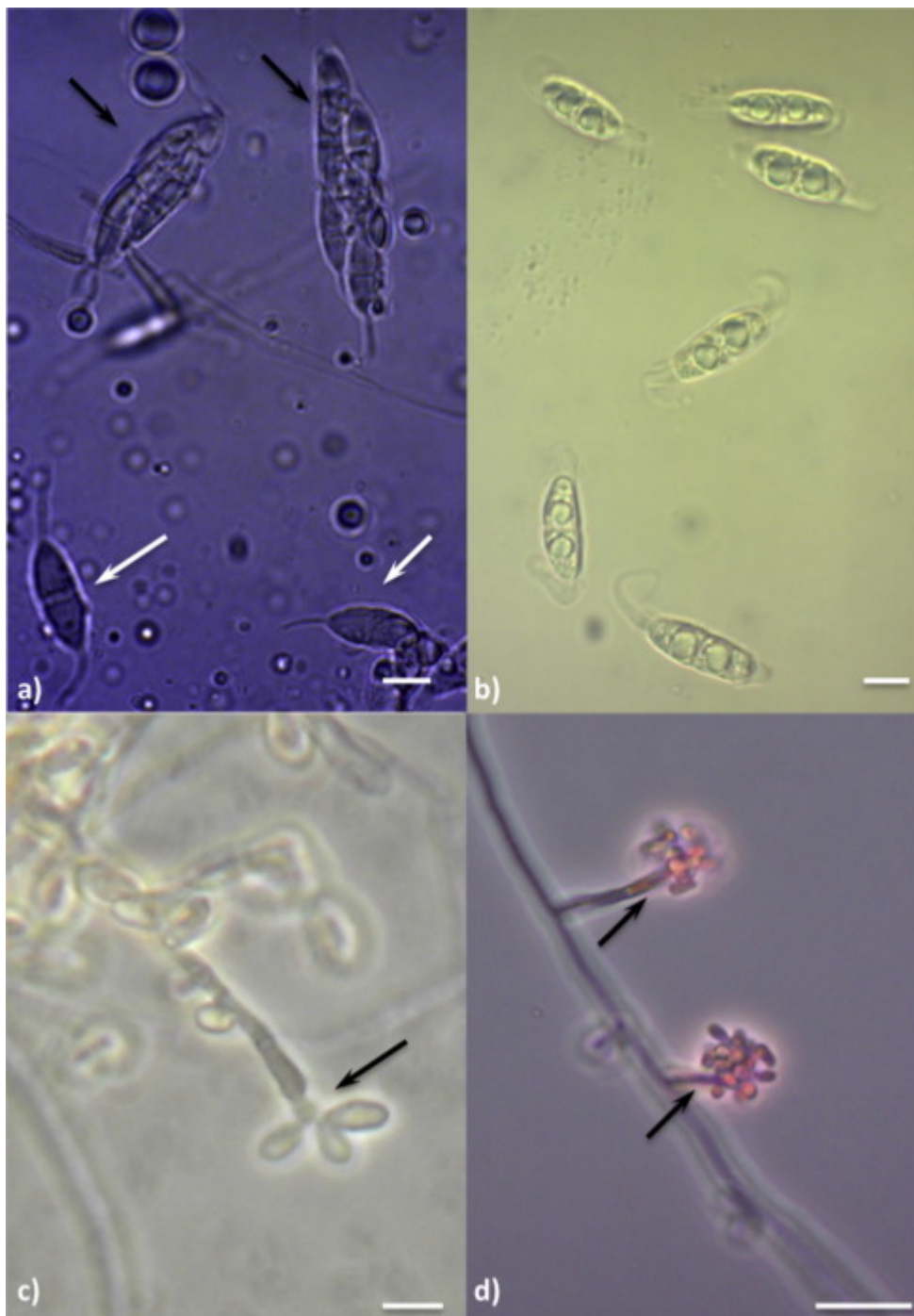


Fig. 3.

Marine fungal strains isolated from wood substrates: (a) *Corollospora maritima*, ASCII (black arrows) and ascospores bi-septate with apical appendages (white arrows); (b) *Ceriosporopsis halima* ascospores with apical appendages; (c) *Scolecobasidium arenarium* phialide (black arrow) bearing conidia; (d) *Exophiala angulospora*, unbranched conidiophores with terminal conidiogenous cells (black arrows). Scale bar = 10 μ m.

Other obligate marine species, which are commonly associated to wood substrates in the Mediterranean Sea (e.g. *Cirrenalia macrocephala*, *Halosphaeria quadriremis*, *Humicola alopallonella*, *Piricauda pelagica*, *Remispora maritima* and *Torpedospora radiata*, [Table 2](#)), were not detected in our study.

Despite the small number of samples we analyzed (25) compared to previous studies (e.g. [Cuomo et al., 1988](#) isolated 75 species on 6462 wood samples from 13 locations) we identified 40 taxa from 5 sampling sites. Moreover, 36 of the 40 taxa we isolated are new records for Italian lignicolous marine fungi. There are two possible explanations for this discrepancy in the number of isolated species per analyzed substrate (recorded in the present study compared to previous studies): first of all, only obligate marine species were recorded before 1998 ([Hyde et al., 1998](#)); secondly, in previous studies, species were not identified by means of molecular techniques. Indeed, the majority of taxa we recorded belonged to the heterogeneous group of the so-called facultative marine species. More specifically, in addition to taxa commonly reported on marine substrates such as *Scolecobasidium arenarium* ([Fig. 3c](#)) and *E. angulospora* ([Fig. 3d](#)), we reported species that used to be considered as mere terrestrial contaminants and are now recognized as an integral part of the marine wood communities ([Rämä et al., 2014](#)), for example *H. haematococca* (recognized as a serious pathogen of sea turtle eggs, [Sarmiento-Ramírez et al., 2010](#)), *P. expansum* (also reported in seawater, [Pindi, 2012](#)), *A. tenuissima* (frequently found as a mangrove endophyte, [Sun et al., 2013](#)), and *C. luteo-olivacea* (recognized by [Rämä et al., 2014](#) as a dominant component of the marine wood-inhabiting mycobiota). Moreover, among these non-obligate species, *C. kleinmondensis*, *G. stilboides* and *Scopulariopsis hibernica*, had not previously been recorded in marine environments. The newly-described species *Capronia kleinmondensis* ([Marinvowitz et al., 2008](#)), observed for the first time in association with leaf, twig litter and senescent flower heads of Proteaceae (a family of plants commonly found in xeric environments), had not previously been reported in other substrates; *G. stilboides* is a worldwide crop pathogen ([Desjardins, 2003](#)); *S. hibernica* is recognized as the etiological agent of grass mold ([Mangan, 1965](#)). All the isolated strains were found to be able to grow on a salty media, thus confirming the hypothesis that these species adapt well to marine environments.

Only a few species were isolated from both substrates (driftwood and submerged wood): two common marine species (*C. maritima* and *Lulwoana* sp.) and a few cosmopolitan species (i.e. *Clonostachys rosea* f. *rosea* and *H. haematococca*). Despite driftwood is more susceptible to aerial and terrestrial fungal contaminants, fungi that live on this substrate are selected mainly because they have to withstand desiccation and increase in salinity levels. On the other hand, fungi on submerged wood must be able to grow in environments with high pressure and low temperatures ([Jones, 2000](#)). In accordance with [Rämä et al. \(2014\)](#), the different mycobiota found on the two substrates show that fungal communities on wood substrates change in response to biotic and abiotic factors, following wood fate through the sea.

The isolation of several strains of Sordariomycetes (42% of analyzed samples) deserves particular attention. Although this order is very common in marine habitats, neither morphological (isolates in pure culture did not produce propagules) nor molecular analyses (similarity was lower than 85%) allowed us to assign them to any species. Isolates of Sordariomycetes sp.1 probably belong to Lulworthiales order; Sordariomycetes sp.2 could represent a new taxa within the *Corollospora* genus; Sordariomycetes sp.3 probably belongs to Halosphaeriaceae family. Further analyses are required to clarify whether they may represent new species.

In addition, 12% of the observed fungi were found to be uncultivable on different substrates, a problem that is common to all marine mycobiota investigations. Recently, this problem was tackled by a number of scientists who tried to develop culture-independent methods to reveal the overall microbial diversity in marine environments ([Manohar and Raghukumar, 2013](#)). Nonetheless, these culture-independent methods suffer from the lack of reference strains available in cultures, which can help to provide correct morphological identifications and reliable barcoding sequences. As [Rämä et al. \(2014\)](#) stressed, this lack makes it impossible to decide whether putative terrestrial taxa found in marine environments are facultative genus or new lineages adapted to marine habitats.

The present study is therefore part of a more extensive project with the aim to provide highly reliable barcoding sequences that can be used in taxonomical analyses ([Gnavi et al., 2014](#)) and publicly accessible strains ([Stackelbrandt et al., 2014](#)). As previously stated, fungi that grow on marine wood have different stresses to overcome, but they also need to be able to use the complex substrates on which they are developing. They therefore possess enzymes that are capable of degrading the complex constituents of wood, such as lignin, even in marine conditions. Due to these enzymes, marine fungi are of particular interest in view of bioremediation of hydrocarbon pollutants ([Harms et al., 2011](#) and [Damare et al., 2012](#)). These valuable properties were confirmed in our study: most of the strains were able to grow on media with hydrocarbons as the sole carbon source. The majority of the isolates performed better in the presence of NaCl, which confirms that they adapt well to marine conditions and that salt can trigger specific metabolic pathways ([Panno et al., 2013](#)). Among the most promising strains, *A. tenuissima*, *G. stilboides*, and *N. exilis* showed the best performances on both substrates. *Alternaria tenuissima* is already known for its ability to grow and degrade hydrocarbons in terrestrial habitats ([Llanos and Kjølner, 1976](#) and [Srivastava et al., 2014](#)); on the contrary, *G. stilboides* and *N. exilis* had not previously been reported to have been found in oil contaminated sites and had never been tested for their degradative abilities. Although *C. kleinmondensis* and *G. nigrescens* had not previously been characterized for their bioremediation potential, in our study they were found to grow and produce a clarification halo on BTZ. Other species (such as *C. halima*, *H. haematococca*, *P. citrinum*, *P. linacinum* and *P. boydii*) known for their biodegradation properties in terrestrial habitats ([Llanos and Kjølner, 1976](#), [Kirk and Gordon, 1988](#), [April et al., 1998](#), [Itah and Essien, 2005](#) and [Al-Nasrawi, 2012](#)) were confirmed to possess the same ability in marine isolates. A final consideration can be made about *C. maritima*, whose strains showed different degradation capabilities. *Corollospora maritima* strain 1 and 2 were isolated on driftwood from “Cinque Terre” MPA and Porto Badisco respectively, while *C. maritima* strain 3, the most active on both hydrocarbon substrates, was isolated in “Berpeggi” MPA from submerged wood. These findings show that different adaptations can occur in marine habitats; collecting fungal strains provides a “treasure chest” of valuable microorganisms.

5. Conclusions

Although driftwood and submerged wood are the most commonly and traditionally studied substrates in marine mycology, the global microbial biodiversity associated to these substrates is far from being fully revealed. A few years ago a large number of studies were based on culture-independent methods and molecular tools; however, more recently there has been an increasing need to go back to a holistic/polyphasic approach that combines molecular analyses and morpho-physiological studies, as the present study does. This requirement arises from the necessity for viable strains that are correctly identified and available for scientific communities. This is particularly useful to fill the gap in barcoding sequences, and more generally for future purposes such as the characterization of new lineages and the development of useful technologies (e.g. bioremediation).

The present study has contributed towards filling gaps in the knowledge on fungal communities on lignicolous substrates in the Mediterranean Sea. Thirty-three species, among which strains that possess a strong biodegradative potential, have been added to the Mediterranean checklist. A mycological collection of marine strains has been created, and is now available in an internationally recognized biological center.

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Appendix A. Supplementary material

SUPPLEMENTARY MATERIAL 1 BLAST results and sequence information

Taxa	MUT accession number	PAV-M accession number	GenBank B	
			% of sequence identity	% of query coverage
<i>Acremonium implicatum</i> (J.C. Gilman & E.V. Abbott) W. Gams	1575	1.012	100	97
<i>Acremonium tubakii</i> W. Gams	3425	1.141	98	94
<i>Acremonium furcatum</i> Moreau & F. Moreau ex Gams	1876	1.065	97	95
<i>Acremonium</i> sp.	3134	1.125	100	100
<i>Alternaria tenuissima</i> (Kunze) Wiltshire	1816	1.036	100	99
<i>Aspergillus protuberus</i> Munt.-Cvetk.	3407	1.166	100	100
<i>Botryotrichum piluliferum</i> Sacc. & Marchal	3142	1.128	99	97
<i>Cadophora luteo-olivacea</i> (J.F.H. Beyma) T.C. Harr. & McNew	1612	1.017	100	99
	2963	1.100	100	100
<i>Capronia kleinmondensis</i> Marinc., M.J. Wingf. & Crous	1591	1.016	98	99
<i>Cladosporium halotolerans</i> Zalar, de Hoog & Gunde-Cim.	3156	1.130	100	99
<i>Corollospora maritima</i> Werdem	1652	1.020	100	99
	1662	1.021	99	100
	1663	1.022	100	99
	3408	1.163	99	100
	3410	1.137	99	100
	3370	1.171A	99	100
<i>Engyodontium album</i> (Limber) de Hoog	2765	1.087	100	99
<i>Exophiala angulosporea</i> Iwatsu, Udagawa & T. Takase	1634	1.019	97	97
<i>Fusarium oxysporum</i> Schltdl.	3293	1.162	100	100
<i>Gibberella avenacea</i> R.J. Cook	2725	1.075	100	99
			100	100
<i>Gibberella fujikuroi</i> (Sawada) Wollenw.	3411	1.139	99	100
			97	100
<i>Gibberella stilboides</i> W.L. Gordon ex C. Booth	1800	1.035		

<i>Gibellulopsis nigrescens</i> (Pethybr.) Zare, W. Gams & Summerb.	2726	1.076	99	100
<i>Haematonectria haematococca</i> (Berk. & Broome) Samuels & Rossman	1863	1.051	99	100
<i>Ilyonectria radicola</i> (Gerlach & L. Nilsson) P. Chaverri & C. Salgado	2723	1.073	100	100
<i>Lulwoana</i> sp. §	1483	1.02	97	98
	3417	1.169	97	98
	3347	1.167	97	100
<i>Niesslia exilis</i> (Alb. & Schwein.) G. Winter	3202	1.157	99	99
<i>Penicillium expansum</i> Link	2954	1.104	100	99
<i>Penicillium citrinum</i> Thom	2727	1.077	100	100
<i>Penicillium</i> sp.	2772	1.089	100	100
<i>Pseudallescheria boydii</i> (Shear) McGinnis, A.A. Padhye & Ajello	3300	1.156	99	98
<i>Scolecobasidium arenarium</i> (Nicot) M.B. Ellis	1723	1.030	99	100
<i>Scopulariopsis hibernica</i> A. Mangan	3137	1.127	99	100
Sordariomycetes sp. 1	1541	1.011	97	100
Sordariomycetes sp. 2	1587	1.013	98	94
			99	93
Sordariomycetes sp. 3	3357	1.171B	81	66
	3395	1.171D	81	65
Sordariomycetes sp. 4	3401	1.171C	100	100
<i>Trichoderma atroviride</i> P. Karst.	3133	1.123	100	100
<i>Trichoderma</i> sp.	1864	1.052	100	100

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Corresponding author. Tel.: +39 0382 984874.

[1](#) Tel.: +39 0382 984874.

[2](#) Tel.: +39 011 6705984.