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Diversity and biotechnological potential of lignicolous marine fungi from Ria de Aveiro

Diversidade e potencial biotecnológico de fungos marinhos lenhícolas da Ria de Aveiro

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Artur Jorge da Costa Peixoto Alves, Professor Auxiliar com Agregação do Departamento de Biologia e CESAM (Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro.

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o júri

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palavras-chave

Fungos marinhos lenhícolas; Diversidade fúngica; Taxonomia; Filogenia; Potencial Biotecnológico; Ria de Aveiro.

Resumo

Apesar de o conhecimento relativo ao grupo ecológico dos fungos marinhos lenhícolas ser ainda escasso, especialmente em Portugal, é sabido que possuem um papel ecológico essencial nos ecossistemas marinhos e estuarinos, nomeadamente na degradação da matéria orgânica presente na madeira e consequente contribuição para o funcionamento do "anel microbiano" aquático que impulsiona os ciclos biogeoquímicos e a manutenção das relações tróficas. Para além do seu contributo ambiental são também uma fonte de compostos com potencial biotecnológico, com aplicações em várias áreas industriais e farmacêuticas.

Neste trabalho, de modo a investigar a ocorrência de fungos marinhos lenhícolas no estuário da Ria de Aveiro, blocos de madeira (Pinus pinaster) foram submersos numa marina, durante um ano. Dezassete espécies de fungos diferentes foram identificadas com sucesso, observando-se uma predominância de espécies pertencentes à família Lulwortiaceae. Através de análises filogenéticas baseadas nas regiões do DNA ribossomal ITS (internal transcribed spacer), LSU (large subunit ribossomal) e SSU (small subunit ribosomal) conjugadas com estudos morfológicos e fisiológicos foram introduzidos dois géneros (Lulworthiopsis e Marinomyces) para acomodar Lulworthia cf. purpurea e Papulaspora halima, respetivamente. De igual modo, Lulworthiopsis maritima sp. nov., Halosphaeria submersa sp. nov. e Zalerion pseudomaritima sp. nov. são propostas como espécies novas.

Para avaliar as suas potencialidades biotecnológicas, isolados de interesse foram selecionados e realizaram-se testes enzimáticos e antibacterianos. Como era esperado de espécies lenhícolas, a maioria dos isolados testaram positivo para as atividades celulolíticas e pectinolíticas. Halosphaeria submersa, M. halima e Z. maritima revelaram um interessante potencial em várias atividades enzimáticas extracelulares. Foram ainda detetadas diferenças na produção enzimática na presença e ausência de sal, sugerindo assim que estas atividades possam ser estimuladas ou inibidas consoante a salinidade. Em relação aos resultados da atividade antibacteriana, os isolados selecionados apresentaram uma maior eficácia contra bactérias Gram-positivo. Lulworthiopsis purpurea e M. halima mostraram atividade antibacteriana contra as bactérias Gram-negativo multirresistentes Pseudomonas aeruginosa e Klebsiella pneumoniae, respetivamente, representando um potencial antibacteriano eficaz no combate a estas espécies patogénicas.

Futuros estudos taxonómicos e biotecnológicos focados em fungos marinhos lenhícolas podem vir a trazer benefícios para diversas áreas de investigação.

keywords

Lignicolous marine fungi; Fungal diversity; Taxonomy; Phylogeny; Biotechnological potential; Ria de Aveiro

abstract

Although knowledge about the ecological group of lignicolous marine fungi is still scarce, especially in Portugal, it is known that they play an essential ecological role in marine and estuarine ecosystems, namely in the degradation of organic matter present in wood and consequent contributing to the functioning of the aquatic “microbial ring” that drives the biogeochemical cycles and the maintenance of trophic relationships. In addition to their environmental contribution, they are also a source of compounds with biotechnological potential, with applications in various industrial and pharmaceutical areas.

In this study, in order to investigate the occurrence of lignicolous marine fungi in the estuary of Ria de Aveiro, blocks of wood (Pinus pinaster) were submerged in a marina, for one year. Seventeen different fungal species were successfully identified, with a high prevalence of species belonging to the family Lulwortiaceae. Through phylogenetic analyses based on the ribosomal ITS (internal transcribed spacer), LSU (large ribosomal subunit) and SSU (small ribosomal subunit) regions combined with morphological and physiological studies, two genera (Lulworthiopsis and Marinomyces) were introduced to accommodate Lulworthia cf. purpurea and Papulaspora halima, respectively. In the same way, Lulworthiopsis maritima sp. nov., Halosphaeria submersa sp. nov. and Zalerion pseudomaritima sp. nov. were proposed as novel species.

To assess their biotechnological potential, isolates of interest were selected and enzymatic and antibacterial tests were carried out. As expected from lignicolous species, most isolates tested positive for cellulolytic and pectinolytic activities. Halosphaeria submersa, M. halima and Z. maritima revealed an interesting potential in several extracellular enzymatic activities. Differences were also detected in enzyme production in the presence and absence of salt, thus proposing that these activities can be stimulated or inhibited depending on the salinity. Regarding the results of antibacterial activity, the selected isolates showed more effective against Gram-positive bacteria. Lulworthiopsis purpurea and M. halima showed antibacterial activity against the multiresistant Gram-negative bacteria of Pseudomonas aeruginosa and Klebsiella pneumoniae, respectively, representing an anti-bacterial potential effective in combating these pathogenic strains.

Future taxonomic and biotechnological studies focused on lignicolous marine fungi may bring benefits to several areas of research.

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Chapter I

General Introduction

"We know what the surface of the moon is better than we know what the surface of the sea floor is."

- James Gardner

General Introduction

Definition of marine fungi

Over the past few decades, several proposals have been made to define what marine fungi are, due to being a group yet to be explored by the scientific community, with many species yet to be discovered and characterized, as well as due to the increased biotechnological interest in studying these microorganisms (Richards et al. 2012; Zhang and Kim, 2012). The first attempt to define what marine fungi are was made by Kohlmeyer and Kohlmeyer (1979), in which the authors created a distinction between "obligate marine fungi" and "facultative marine fungi": "obligate" includes fungi that can only grow and sporulate in a marine environment and "facultative" includes species capable of growing and sporulating, not exclusively in the marine environment, but also in freshwater and/or terrestrial environments. In 2016, Pang et al. suggested a more up-to-date change to the definition of marine fungi: “any fungus should be considered marine if it is collected repeatedly from marine habitats, where they demonstrate being able to grow and sporulate in these environmental conditions and/or create symbiotic relationships with other marine organisms and/or if they prove capable of being metabolically active, adapted and able to evolve genetically in marine habitats”. This new approach is much more wide-ranging and therefore included more species in this ecological group (Sarma, 2019).

Diversity and distribution

Of the fungal species already described by the scientific community, only 1% are integrated into the group of marine fungi (Sarma, 2019). It is estimated that there are around 10 000 species of marine fungi (Jones, 2011), but the most up-to-date number of described species is only 1 257, classified in 539 genera, 168 families, 74 orders, 20 classes and 5 phyla (Jones et al. 2019). Some lack of information related to marine fungal biodiversity may also be associated with the fact that, before 1998, many mycologists only documented "obligate" species, with everything else being considered “terrestrial contamination” without any involvement in the marine ecology (Hyde et al. 1998). However, more species are being discovered due to the increased interest of this ecological group and also due to the development of new molecular techniques capable of identifying non-cultivable species or very difficult to cultivate in the laboratory (Hongsanan et al. 2018; Jones et al. 2019; Sarma,

2019). At present, the most represented phyla among marine fungi are Ascomycota and Basidiomycota, including more than 90% of the total described diversity of marine fungi (Hyde et al. 2000; Jones and Pang, 2012; Jones et al. 2019). *Halosphaeriaceae* is the most numerous family; and *Aspergillus* and *Penicillium*, common soil-associated fungal genera that are commonly found in marine environments (Jones et al. 2015).

Concerning the distribution of this ecological group, it is to be agreed that these microorganisms are present in all types of marine ecosystems, all across the world, from the poles to the tropics, present between coastal regions and deeper ocean areas, although some taxa are geographically restricted (Fell & Newell, 1998; Hyde et al. 1998). Also they demonstrate to be a very adaptive group, surviving in habitats with extreme conditions, such as deep sea habitats with extreme hydrostatic pressure and low temperature conditions, like Mariana trench, the deepest place known on the ocean floor (Xu et al. 2014) or hydrothermal ecosystems (Van Dover et al 2007; Le Calvez et al. 2009); aquatic sites with high concentrations of salinity, like the Dead Sea (Buchalo et al. 1998; Raghukumar, 2008); and icecold temperature waters in the Arctic and Antarctica (Loque et al. 2010; Hassett and Gradinger, 2016). Also present in not so extreme habitats, these species are very often found in on beach sands, salt marshes, mangroves, in brackish or estuarine waters and even freely in seawater (Janso et al. 2005; Figueira and Barata, 2007; Gonçalves et al. 2019a, 2019b, 2020; Jones et al. 2019). In order to survive in so many aquatic niches, these microorganisms have adapted to live in the conditions of the sea, which are quite different from the conditions experienced by terrestrial fungi, namely the marine agitation, availability of oxygen, hydrostatic and osmotic pressures, concentration of hydrogen ions, salinity, competition against other micro or macroorganisms and the availability of hosts/physical support (Booth and Kenkel, 1986; Pang et al. 2011; Sergeeva, and Kopytina, 2014). Something that may have been a determining factor in the spread of fungal diversity is the dispersion of spores by sea currents, allowing the colonization of individuals of the same species in different locations (Azevedo et al. 2017). Within these aquatic ecosystems, various substrates are used as a physical base and support for the growth and proliferation of the marine fungi, such as marine sediment, macro and microalgae, sponges, tunicates, coral reefs, animal calcareous shells, rotten leaves and roots in mangroves, roots and leaves of intertidal plants, invertebrate guts, woody substrates such as driftwoods and submerged woods, among many

others (Kohlmeyer and Kohlmeyer, 1979; Hyde at al. 1998; Bugni and Ireland, 2004; Richards et al. 2012; Garzoli et al. 2015; Gonçalves et al. 2019b; Sarma, 2019).

Ecological activities

In addition to being ubiquitous, fungi are a group with an impact on the ecology of aquatic ecosystems, being an important element in the recycling of nutrients in the environment (Hyde et al. 1998), also contributing to the maintenance of the energy flow to higher trophic levels (Jones et al. 2019), serving as the base and sustain of the ocean´s food web. Marine fungi ensure the normal functioning of the ecosystem and enable the existence of marine biodiversity through symbiotic relationships, as saprobes, parasites, endobionts and/or mutualists (Jones et al. 2019).

Throughout their evolution, fungi have lost the ability to phagocyte food, developed a chitinous cell wall and feed osmotrophically, through the input of environmental nutrients. The use of secondary metabolism and secretion of digestive enzymes is an adaptation developed to break down complex molecules and to facilitate access to nutrients (Richards et al. 2012). Marine fungi that absorb extracellular organic substances to feed are called saprobes, which are normally associated with the decomposition of organic matter (Kohlmeyer and Kohlmeyer, 1979). These have a crucial role in the decomposition of micro and macroalgae, plants, dead animals and calcareous structures (bones or shells), substrates from which fungi release nutrients, part of which is released into the environment, enabling the use of these molecules by other marine organisms (Raghukumar, 2008). A great example of this ecological function is observed in mangrove environments, where this group is not only quite predominant but determinant in the decomposition of plants (Bremer, 1995; Pang et al. 2011).

Some are considered parasites of many marine organisms, such as fish, algae or invertebrates (Hyde et al. 1998; Richards et al. 2012; Raghukumar, 2017), with Chytridiomycota being a phylum known for having phytoplanktonic parasitic species, responsible for the decreasing in diatomaceous populations, changing the composition of the phytoplanktonic community and at the same time transferring more nutrients to zooplanktonic organisms (Kagami et al. 2007a, 2007b; Rasconi et al. 2012; Hanic et al. 2009; Gutierrez et al. 2016). The endophytic fungi installing their selves inside the host without harming it, which thus serves as support for the growth of the fungi. If a mutual relationship is established, the fungi can bring several

benefits to the host, like protection against polluting agents and pathogenic agents (whether bacteria or other harmful fungi) as they can be useful in the development of the host and even improve their chances of surviving and thriving (Barrow et al. 2008; Debbah et al. 2011, 2012; Hom and Murray, 2014).

Lignicolous marine fungi

Whether for support or to obtain energy and organic matter, colonization of wood is carried out by lignicolous marine fungi and this ecological interaction has been a focus of interest (Kohlmeyer and Kohlmeyer, 1979; Sarma and Hyde, 2001; Jones et al. 2019; Garzoli et al. 2015; Rämä et al. 2016; Raghukumar, 2017). For long, lignicolous marine fungi have been known for being the major decomposing agents of wooden substrates, like driftwood, submerged baits and herbaceous plants (Kohlmeyer et al. 1995; Shearer et al. 2007, Garzoli et al. 2015), due to their enzymatic activity capable of degrading lignocellulose (Eaton and Hale, 1993; Panno et al. 2013). According to Raghukumar (2017), these lignicolous microorganisms can be ecologically divided in endophytic during the normal life cycle of the host that, after death, remain in the substrate and enhance its decomposition or only colonize in decomposing bodies/substrates.

Some authors classify the diversity of these fungi as low, taking into account the small number of isolated species for the high number of samplings carried out (Cuomo et al. 1988), while others recognize the wealth fungal diversity around these substrates (Abdel-Wahab, 2011; Garzoli et al. 2015; Rämä et al. 2016; Jones et al. 2019). In any case, it is agreed that studies related to fungal diversity in wood are scarce, especially in submerged wood. From what is known, the Ascomycota phylum is the most prevalent (Garzoli et al. 2015) and Raghukumar (2017) estimates that there are about 490 known species affiliated with wood, 190 being from drift and submerged wood; Abdel-Wahab (2011) even affirms that the fungal communities found in allochthonous wood are different from the mangrove communities. In the notorious work of Kohlmeyer and Kohlmeyer (1979), four species were collected from wood submerged in depth: *Bathyascus vermisporus, Oceanitis scuticella, Allescheriella bathygena* and *Periconia abyssa. Lulworthia medusa* was the most frequent species in a wood bait study done in the Italian coast (Montemartini, 1979), where there also were recorded species like *Trichocladium achrasporum, Halosphaeria maritima, Nia vibrissa, Humicola alopallonella, Papulaspora halima, Corollospora maritima* and *Cirrenalia*

macrocephala, some registered for the first time in Mediterranean waters. Same species and genera were found in wood panels in a harbour (also in Italy), *Lulworthia* sp. confirming it´s predominance among wooden substrates, along with *C. maritima* (Grasso et al. 1985, 1990). In Cuomo et al. (1988), 42 of 53 species of sampled marine fungi were lignicolous "growing on wood and other cellulosic materials", along 13 sampling sites in the Mediterranean Sea. In this one, *C. maritima* and *Halosphaeriopsis mediosetigera* were, by far, the most present fungi, both in drift and submerged wood substrates. All the other species were the same as previous studies on this sea, suggesting that diversity in this marine area is similar, only with changes in the abundance of certain key species. Garzoli et al. 2015 also contributed to the enrichment of knowledge regarding the diversity of lignicolous fungi, obtaining species already expected and others that had not been registered with such abundance before, such as *Zalerion* sp., *Penicillium expansum*, *Haematonectria haematococca* and *Alternaria tenuissima.* Yet, Raghukumar (2017) mentions *Halosphaeriaceae* as the most observable family among allochthonous wooden samples and provides an extensive list of species occurring in mangrove systems, which differ from the allochthonous ones, although *Antennospora quadricornuta, Lignincola laevis* and *Lulworthia* sp. (among others) are common in both substrates.

This group of lignicolous fungi has a biotechnological potential and it is studied for many industrial and pharmaceutical applications (Huang et al. 2001; Bugni and Ireland, 2004; Raghukumar et al. 2004, 2008; Lin et al. 2005; Singh and Singh, 2014; Liu and Kokare, 2017) which will be addressed and discussed in Chapter III of this Dissertation.

Studies in Portugal

Despite its extensive seacoast and numerous saline habitats, knowledge of Portuguese marine fungi is still scarce, especially regarding the lignicolous ones (Sridhar et al. 2012). Yet, there is already some bibliography capable of serving as a basis to describe the Portuguese lignicolous fungi.

Isolated from dead culms of *Spartina maritima* submerged for six months in the Mira River estuary, Barata et al. (1997) discovered and described a novel species of marine fungi, *Nia globospora*. Barata (2006) also carried out an annual collection of stems from *S. maritima* that underwent different conditions of submersion in the intertidal zone of the salt marsh ecosystem of the River Mira, which resulted in the identification of 26 marine fungi, of

which 24 were the first records of those species in Portugal and one was proposed as a novel species.

In Figueira and Barata (2007), the diversity present in wooden substrates on two sandy beaches from the west coast (Cascais and Sintra) was studied. Another 15 new species were added to the Portuguese bibliography, 35 (in total) having been identified, of which only 11 of them were represented in both places. This study represents the first records of fungal marine diversity on Portuguese beaches and also on driftwood, of which *Halosphaeriopsis mediosetigera*, *Lignincola laevis* and *Lulworthia fucicola* were observed only in this substrate.

The studies carried out by Azevedo et al. (2010, 2011, 2012, 2017, 2018) came to contribute a lot to unravel the diversity of Portuguese marine fungi. First, 288 baits were placed in two marinas on the western coast (Cascais and Sesimbra), on which 26 taxa were identified, 10 novel in Portugal and 2 novel at European level (Azevedo et al. 2010). In Azevedo et al. 2011, the materials and methods were the same, however the change in incubation on moist chambers caused more fungal isolates to grow, observing a higher population density, with more species present in each bait. In another study, driftwoods and stems of various plant species were placed on four Portuguese Atlantic beaches (Vagueira, Cascais, Meco and Vila Nova de Mil Fontes), from which 56 taxa were recorded, whose occurrences by species strongly depended on the type of substrate. *Corollospora maritima* was present in almost all substrates and the stems of *S. maritima* were the most colonized and had the greatest diversity of lignicolous species (Azevedo et al. 2012). The work of these researchers also contributed to the identification of a novel species, namely *Lulworthia atlantica* (Azevedo et al. 2017) and in the morphological and phylogenetic characterizations of *Nia vibrissa* (Azevedo et al. 2018). In salt marshes in Castro Marim and Ria de Aveiro, Calado (2016) collected 195 live plants of *S. maritima* and the results coincided with those observed in Azevedo et al. (2011), with new records of several species of marine fungi associated to *S. maritima*.

Sridhar et al. 2012 collected 350 pieces of wood waste and 140 pieces of seaweed scraps from 7 beaches on the west coast of Portugal (Moledo, Afife, Carreço, Foz do Neiva, Apúlia, Costa Nova and Mira). Thirty-six lignicolous fungi taxa and 29 algicolous fungi taxa were identified, with the wooden substrate showing the greatest diversity and abundance of isolates.

More recently, Gonçalves et al. (2019a, 2019b) recent works have come to improve and increase knowledge regarding the diversity of some lignicolous marine fungi, both from the marine coast and the estuary of Ria de Aveiro. Gonçalves et al. (2019a) described three novel *Neocamarosporium* species associated with *Halimione portulacoides*, another halophyte as *S. maritima*, and Gonçalves et al. (2019b) also reported *Penicillium terrigenum*, *P. chrysogenum*, *P. sanguifluum* and *P. oxalicum* on driftwood collected in some Portuguese beaches

General Objectives

The overall objective of the present study was to increase our knowledge about the diversity of lignicolous marine fungi in Portugal, specifically in Ria de Aveiro estuary, as well as to assess their bioactivities with biotechnological potential.

The specific aims were 1) describe the diversity of lignicolous marine fungi present in blocks of wood submerged during 1 year in a marina in the estuary Ria de Aveiro, 2) test the enzymatic activity of some isolates that were selected from this collection and 3) test the antibacterial activity of the same selected isolates.

The present dissertation is organized as follows:

Chapter I: General Introduction on marine fungi, it´s definition, diversity and distribution, ecological role in the marine ecosystem, lignicolous marine fungi approach and studies carried out in Portugal;

Chapter II: "Diversity of marine fungi associated to wood baits in the estuary Ria de Aveiro with description of *Lulworthiopsis purpurea* gen. et comb. nov., *Lulworthiopsis maritima* sp. nov., *Marinomyces halima* gen. comb. nov., *Halosphaeria submersa* sp. nov., and *Zalerion pseudomaritima* sp. nov.";

Chapter III: Screening of enzymatic and antibacterial activity of marine lignicolous fungi;

Chapter IV: Final considerations and Future perspectives.

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Chapter II

Diversity of marine fungi associated to wood baits in the estuary Ria de Aveiro with description of *Lulworthiopsis purpurea* gen. et comb. nov., *Lulworthiopsis maritima* sp. nov., *Marinomyces halima* gen. comb. nov., *Halosphaeria submersa* sp. nov., and *Zalerion pseudomaritima* sp. nov.

Diversity of marine fungi associated to wood baits in the estuary Ria de Aveiro with description of *Lulworthiopsis purpurea* gen. et comb. nov., *Lulworthiopsis maritima* sp. nov., *Marinomyces halima* gen. comb. nov., *Halosphaeria submersa* sp. nov., and *Zalerion pseudomaritima* sp. nov.

Abstract

Lignicolous marine fungi are a particular group of microorganisms that are typically found in mangroves, salt marshes and estuaries, normally associated to driftwood or submerged wood. During investigations of lignicolous fungi occurring in the estuary Ria de Aveiro, Portugal, wood baits were submerged in a marina for one year. Seventeen distinct marine fungal species were identified with the most abundant taxa belonging to the family *Lulworthiaceae*. Through single and multilocus phylogenies based on sequences of the internal transcribed spacer (ITS) region, large subunit (LSU) and small subunit (SSU) of the ribossomal RNA gene cluster and in conjunction with morphological and physiological data we propose *Lulworthiopsis* and *Marinomyces* as new genera to accommodate *Lulworthia* cf. *purpurea*, as *Lulworthiosis purpurea*, a species related to *Lulworthia fucicola*, and *Papulaspora halima*, as *Marinomyces halima*, a species that at the family/genus level has been somehow confusing. Also, *Lulworthiopsis maritima* sp. nov., *Halosphaeria submersa* sp. nov. and *Zalerion pseudomaritima* sp. nov. are proposed as novel species.

Keywords: Estuary; *Halosphaeriaceae*; Lignicolous fungi; *Lulworthiaceae*; Taxonomy

1. Introduction

According to studies by Hawksworth and Lucking (2017), there are between 120,000 to 143,273 official species of fungi, of which only 1,281 are marine species (Jones et al. 2019; www.marinefungi.org). Although the knowledge related to this group is still scarce, compared to terrestrial fungi and other marine microorganisms (Jones and Richards 2011; Raghukumar, 2017), it is known that they are distributed in several marine ecosystems (Hawksworth and Lucking, 2017; Raghukumar, 2017; Morales *et* al. 2019). They can be

found not only in coastal marine environments, but as well in mangroves, salterns and even in estuarine waters (Gonçalves *et* al. 2019a, 2019b, 2020). Worldwide, the distribution of marine fungi ranges from the Arctic (Rämä *et* al. 2017) to the tropical regions (Jones and Pang, 2012), with some species able to survive in extreme environmental conditions, such as anoxic deep seafloor (Orsi *et* al. 2013), in waters with hypersalinity (Raghukumar, 2008) and in hydrothermal vent ecosystems (Xu *et* al. 2017).

They can establish ecological relations, like parasitism or symbiosis, with other marine organisms, such as algae (Raghukumar, 2008; Kohlmeyer and Kohlmeyer, 2013; Gonçalves *et* al. 2020), sponges (Höller *et* al. 2000); corals (Amend *et* al. 2012) and tunicates (Shaala and Youssef, 2015). Also, they can be found in other substrates, like marine sediments (Raghukumar, 2008), shells of calcareous animals (Raghukumar and Damare, 2008) and wood detritus (Hyde *et* al. 2000; Kohlmeyer and Kohlmeyer, 2013).

Wood in marine environments is originated from different ecosystems, such as salt marshes, mangroves and terrestrial habitats, and once at sea, it is colonized by marine organisms including fungi. Colonization by fungal marine species is already documented by several studies (Jones, 1968; Garzoli *et* al.2015; Rämä *et* al. 2016). In both aquatic and terrestrial environments, wood is a source of energy and serves as a physical support for many species (Eriksson *et* al. 1990), although there are more studies focused on terrestrial communities compared to aquatic (Rämä *et* al. 2016). Fungi are the main agents of enzymatic degradation of lignocellulose present in drift or submerged wood (Hyde *et* al. 1998), as well as in pilling's, trunks, mangrove roots and even in wooden boats (Shearer *et* al. 2007). In association with wood, they are capable of producing laccases and peroxidases, enzymes with the potential to degrade lignin and some recalcitrant environmental pollutants and therefore they can be used in bioremediation (Haritash and Kaushik, 2009; Garzoli *et* al. 2015). They also may contribute to a recycling of nutrients trapped in the wooden substrate, such as fatty acids, essential for the maturation of some marine organisms (Phillips *et* al. 1984).

Studies on the diversity of lignicolous fungi found in submerged wood are scarce. Some studies in Mediterranean Sea, especially in the Italian Peninsula by Garzoli et al. 2015, contributed to update the number of registered taxa associated with wooden substrates. The phylum Ascomycota was the most common found. The most frequent species associated with submerged wood or driftwood belong to the genera *Acremonium*, *Alternaria*,

Aspergillus, *Ceriosporopsis*, *Cirrenalia*, *Cladosporium*, *Corollospora*, *Fusarium*, *Gibberella*, *Halosphaeria*, *Lulworthia*, *Periconia*, *Penicillium*, *Stemphylium*, *Trichoderma* and *Zalerion* (Jones *et* al. 1972; Montemartini, 1979; Grasso *et* al. 1985; Cuomo *et* al. 1988; Grasso *et* al. 1990; Garzoli *et* al. 2015). Studies on the diversity of lignicolous fungi from Portuguese marine environments are limited. Results from Azevedo et al. (2010, 2011) and Gonçalves et al. (2019c) corroborate the previous findings and found more species from well-known genera capable of colonizing wood substrates.

The aim of this study was to explore the diversity of wood-colonizing marine fungi in Ria de Aveiro, using a strategy based on the submersion of wood baits. Here, we also report the morphological, cultural and phylogenetic characterization of two novel genera and three novel species of lignicolous ascomycetes.

2. Materials and Methods

2.1. Collection and isolation

Ten wood block baits (*Pinus pinaster*) were submerged to 3 meters deep in a marina, located in Ria de Aveiro (40°37'48''N 8°43'58''W). The baits remained under water for 1 year. Afterwards, the samples were recovered, placed in sterile containers and preserved in the cold (4°C), until fungal isolation. The surface of the wood baits was scraped with a knife, eliminating marine crusting organisms such as sponges, algae and tunicates. Then, the wood baits were cleaned with autoclaved filtered saline water. After surface cleaning, each wood sample was cut into small pieces and sterilized with 96 % ethanol for 1 minute and rinsed twice in sterile water for 1 minute. Fungal isolations were made by placing the small pieces of wood baits in potato dextrose agar (PDA) medium containing 3% sea salts (Sigma-Aldrich). Streptomycin and tetracycline, at final concentrations of 100 mg L^{-1}, were added to PDA medium to inhibit the growth of bacteria. Five replicates of PDA plates were used for each wood block bait, making a total of 50 plates. The plates were incubated at 25°C and checked daily for fungal growth. Distinct fungal colonies were then transferred to new PDA plates for further isolation and purification.

2.2. DNA isolation, amplification and analyses

Genomic DNA was extracted from fresh mycelium of cultures growing on PDA following the protocol described by Möller *et al*. 1992. Microsatellite-primed PCR (MSP-PCR) with

GTG_5 primer (5´-GTGGTGGTGGTGGTG-3´) was used for molecular typing of all isolates according to Alves *et al.* 2007. Analysis of the genetic fingerprinting patterns was performed with GelCompar II software (Applied Maths). The Pearson correlation coefficient was applied, and cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The resulting dendrograms were analyzed in order to obtain groups of isolates with at least 80 % similarity. This value corresponds to the reproducibility from which groups of isolates are formed, i.e. the reproducibility was calculated previously in other studies, as the mean value of the reproducibility for the primers used in the MSP-PCR (Santos and Phillips, 2009). Therefore isolates belonging to the same species were typically clustered together at similarity values greater than 80 % and considered as the same taxonomic affiliation. For each group, representative isolates were selected randomly and subjected to PCR amplification of the ITS region of the rDNA using primers ITS1 and ITS4 (White et al., 1990) as described by Alves *et al*. 2004. For some isolates additional molecular markers were used, such as, small and large ribosomal subunit (18S and 28S) using a NS1/NS8 and LR1/LR12 primers sets, respectively, with the following cycling conditions: initial denaturation for 5 minutes at 95ºC, 30 cycles were performed at 94ºC for 30 seconds, followed by the annealing step at 50ºC for 30 seconds and extension at 72ºC for 1 minute and 30 seconds, with a final elongation step at 72ºC for 10 minutes. The amplified PCR fragments were purified with the NZYGelpure kit (NZYTech, Lisbon, Portugal) before being sent to GATC Biotech (Cologne, Germany) for sequencing. The nucleotide sequence analyses were performed with FinchTV version 1.4.0 (Geospiza, Seattle, WA, USA). A BLASTn search against the nucleotide collection (nr/nt) database using the ITS, 18S and 28S sequences was carried out to determine the closest matching sequences, which were added to the sequence alignment. Information from the representative isolates was used to associate taxonomic affiliation to all isolates from the collection. Sequences were aligned with ClustalX version 2.1 (Thompson *et al.* 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). Alignments were checked and edited with BioEdit Alignment Editor version 7.2.5 (Hall, 1999). Phylogenetic analyses were done with MEGA7 version 7.0 (Kumar *et al.* 2016). All gaps were included in the analyses. MEGA7 was also used to determine the best substitution model to be used to build the maximum-

likelihood (ML) tree. ML analysis was performed on a neighbour-joining (NJ) starting tree automatically generated by the software. Nearest-neighbour-interchange was used as the heuristic method for tree inference with 1000 bootstrap replicates. Maximum parsimony (MP) analyses were performed with PAUP 4.0b10 (Swofford, 2003). All characters were unordered and of equal weight, and gaps were treated as missing data. The heuristic search option with 100 random taxon additions and subtree pruning and regrafting (SPR) method as the branch swapping algorithm were applied. Bayesian inference (BI) was performed using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003). Four MCMC chains were set to run for 10 M generations, sampling every 100th generation for a total of 10,000 trees. The first 1000 trees were eliminated from the further analysis. The remaining were used to generate a majority-rule consensus tree and calculate the posterior probabilities (PP). Trees were visualized with TreeView (Page 1996). The sequences generated in this study were deposited in GenBank and taxonomic novelties in MycoBank. Alignment and tree were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S25978?x-access-code=b07e77a43e642da9a4c46524cdb50575&format=html).

2.3. Morphology and growth studies

The new species identified through phylogenetic analyses were observed with a SMZ1500 stereoscopic microscope (Nikon, Japan) and a Nikon Eclipse 80i microscope (Nikon, Japan). Photographs and measurements from fungal structures mounted in 100% lactic acid were taken with a Nikon DSRi1 camera (Nikon, Japan) and the NIS-Elements D program (Nikon, Japan). Colony characters, pigment production and morphological descriptions based on cultures sporulating were registered for all new species. For the isolates CMG 54 and CMG 55 after 1 month of growth on potato dextrose agar (PDA) and corn meal agar (CMA), incubated at 25°C. For CMG 64 and CMG 65 after 15 days of growth on PDA and CMA, incubated at 25°C. For CMG 53 after 15 days of growth on PDA, CMA, malt extract agar (MEA) and synthetic low nutrient agar (SNA), incubated at 25°C. Colony colours (obverse and reverse) were assessed according to the colour charts of Rayner (1970).

For the new species described, temperature growth studies were performed in the culture media described above. Three replicate plates per isolate were incubated at 5, 10, 15, 20, 25, 30 and 35°C in the dark and the colony diameter was measured after 15 days and 1 month.

3. Results

3.1. Diversity of fungal isolates

This study assessed the diversity of the fungal species colonizing wood baits in an estuarine environment. A total of 212 fungal isolates were obtained from wood baits (n =10).

Molecular typing of the fungal collection using MSP-PCR yielded 101 representative isolates for which sequences of the ITS rRNA region were obtained. BLASTn searches against the nucleotide collection (nr/nt) database unambiguously affiliated the isolates to 10 distinct families (Table 1). Among the different taxa identified, the majority of fungal species found belonged to the family *Lulworthiaceae* (92.4%, n = 196), in the samples of wood baits from Ria de Aveiro, followed by *Aspergillaceae* (1.4%, n= 3), *Cladosporiaceae* (1.4%, n = 3), *Hypocreaceae* (1.4%, n = 3), and *Halosphaeriaceae* (0.9%, n = 2).

Table 1. Distribution of the 10 families from wood baits samples submerged in estuary Ria de Aveiro.

Species	Number of isolates	Percentage
Apiosporaceae	1	0.5%
Aspergillaceae	3	1.4%
Cladosporiaceae	3	1.4%
Halosphaeriaceae	2	0.9%
Hypocreaceae	3	1.4%
Lulworthiaceae	196	92.4%
Nectriaceae	1	0.5%
Niessliaseae	1	0.5%
Pleosporaceae	1	0.5%
Sporocadaceae	1	0.5%

From this collection, 14 different marine fungi species were identified (Table 2). In the family *Lulworthiaceae*, we obtained 152 isolates (72%) of *Lulworthia* cf. *purpurea*, 23 isolates (10.8%) of *Papulaspora halima*, 12 isolates (6%) of a *Lulworthia* sp., 5 isolates (2.4%) of *Zalerion maritima* and 4 isolates (1.9 %) of a *Zalerion* sp.

Besides *Lulworthiaceae* species, we obtained *Arthrinium kogelbergense*, *Cladosporium cladosporioides*, *Corollospora maritima*, *Halosphaeria* sp., *Fusarium oxysporum*,

Paradendryphiella salina, Penicillium antarcticum, P. commune, Pestalotiopsis australis, Sedecimiella taiwanensis, Trichoderma atroviride, T. harzianum and *T. viride*.
Some of these isolates that have not been identified at the species level may eventually represent new species.

Table 2. Distribution of the 17 species from wood baits samples submerged in estuary Ria de Aveiro.

Species	Number of isolates	Percentage
Lulworthia sp.	12	6%
Lulworthia cf. *purpurea*	152	72%
Arthrinium kogelbergense	1	0.5%
Cladosporium cladosporioides	3	1.4%
Corollospora maritima	1	0.5%
Fusarium oxysporum	1	0.5%
Halosphaeria sp.	1	0.5%
Papulaspora halima	23	10.8%
Paradendryphiella salina	1	0.5%
Penicillium antarcticum	1	0.5%
Penicillium commune	2	0.9%
Pestalotiopsis australis	1	0.5%
Sedecimiella taiwanensis	1	0.5%
Trichoderma atroviride	1	0.5%
Trichoderma harzianum	1	0.5%
Trichoderma viride	1	0.5%
Zalerion sp.	4	1.9%
Zalerion maritima	5	2.4%

3.2. Phylogenetic analysis

As mentioned above, the isolates that could not be affiliated to any of the currently known species may represent potential novel taxa. Thus, representative isolates of *Halosphaeria* sp. (CMG 53), *Lulworthia* sp. (CMG 54 and CMG55) and *Zalerion* sp. (CMG 64 and CMG 65) were selected to be further characterised. BLASTn searches against the NCBI nucleotide database using the ITS sequences for CMG 53 retrieved *Remispora quadri-remis* (GenBank accession no. MH858170; Identities 499/569 (88%), 4 gaps), *R. stellata* (GenBank accession no. NR_160085; Identities 496/567 (87%), 3 gaps) and *R. stellata* (GenBank accession no.

MH857977; Identities 496/567 (87%), 3 gaps). The LSU gene was also sequenced to confirm the phylogenetic placement in *Halosphaeria*. The highest similarities using the LSU sequence were *R. maritima* (GenBank accession no. HQ111013; Identities 990/1023 (97%), 3 gaps), *Nais inornata* (GenBank accession no. AF539476; Identities 988/1023 (97%), 3 gaps) and *Naufragella spinibarbata* (GenBank accession no. HQ111034; Identities 984/1028 (96%), 8 gaps). Therefore, sequences (ITS + LSU) of CMG 53 were aligned with those of several related *Halosphaeriaceae* species (Table 3). The alignment of the ITS + LSU contained 18 sequences (including the outgroup), and there was a total of 1809 positions in the final dataset. In ML phylogenetic tree (Fig. 1), the novel isolate clustered in a clade that received high (100%) bootstrap support with high PP values (1.00) within the genus *Halosphaeria* with a close relationship with *H. quadri-remis* and *H. stellata*. Single trees (LSU and ITS) are given in Fig. S1 and S2.

Table 3. List of *Halosphaeriaceae* isolates used in this study.

Genera	Species	Acession Number	Host/Substrate	Country	GenBank Acessions	
					ITS	LSU
Corollospora	*Corollospora angusta*	AFTOL-ID 5010	-	-	-	FJ176900
		NBRC 32100	-	Japan	JN943380	JN941476
	Corollospora cinnamomea	NBRC 32125	Beach sand	Singapore	AB361023	JN941479
		NBRC 32126	Beach sand	Singapore	-	JN941480
		NBRC 32103	-	Japan	-	JN941481
	Corollospora filiformis	PP3905	-	-	-	AF491256
	Corollospora fusca	NBRC 32109	-	Singapore	JN943384	JN941485
		NBRC 32108	-	Japan	-	JN941484
	Corollospora gracilis	NBRC 32111	Sea foam	Japan	JN943386	JN941487
		NBRC 32110	Sea foam	Japan	AB361025	JN941486
	Corollospora intermedia	PP3910	-	-	-	AF491258
	Corollospora lacera	NBRC 32122	-	Japan	-	JN941489
		NBRC 32121	-	Japan	-	JN941488
	Corollospora luteola	NBRC 31316	sea foam	Japan	-	JN941491
		NBRC 31315*	sea foam	Japan	-	JN941490
	Corollospora marina	AFTOL-ID 5008	-	-	-	FJ176898
	Corollospora maritima	NBRC 32117	-	Japan	JN943388	JN941492
		MD 825	Decayed driftwood	Egypt	AB361032	AB361012
		MD 831	Decayed driftwood	Egypt	AB361028	AB361010
		CMG 52	**Submerged wood**	**Portugal**	**MT235714**	**MT232383**
	Corollospora portsaidica	MD 832	Decayed driftwood	Egypt	AB361031	AB361016
		NBRC 32118	-	Japan	JN943387	JN941493
	Corollospora pseudopulchella	NBRC 32113	-	Japan	-	JN941495

		NBRC 32112	-	Japan	-	JN941494
	Corollospora pulchella	NBRC 32123	-	Japan	-	JN941496
		NBRC 32124	-	Japan	-	JN941497
	Corollospora quinqueseptata	NBRC 32114	-	Japan	-	JN941498
		NBRC 32115	-	Japan	-	JN941499
	Corollospora ramulosa	CBS 398.65*	-	USA	NR_160104	NG_064049
Halosphaeria	*Halosphaeria appendiculata*	CBS 197.60	-	USA	-	MH869504
		NTOU4004	Driftwood	-	-	KX686782
	Halosphaeria cucullata	NTOU778	-	-	-	KX686802
	Halosphaeria galerita	BCC33500	Decayed driftwood	Denmark	-	HQ111014
		PP5577	Decayed driftwood	-	-	HQ111015
	Halosphaeria hamata	CBS 145.60	-	USA	MH857929	MH869473
	Halosphaeria maritima	BBH28309	Decayed driftwood	Denmark	-	HQ111012
		BCC33517	Decayed driftwood	Denmark	-	HQ111013
	Halosphaeria pileata	BBH28306	Decayed driftwood	Denmark	-	HQ111022
		BBH28305	Decayed driftwood	Denmark	-	HQ111021
	Halosphaeria quadri-remis	CBS 334.62	timber	-	MH858170	MH869762
	Halosphaeria quadricornuta	NTOU3763	-	-	-	KX686757
		GR89	-	USA	-	EF383130
	Halosphaeria salina	NTOU3998	-	-	-	KX686774
	Halosphaeria spitsbergenensis	CY5279	Decayed driftwood	Norway	-	HQ111011
	Halosphaeria stellata	CBS 258.60*	-	USA	NR_160085	NG_064025
	Halosphaeria submersa	**MUM 20.48/CMG 53***	**Submerged wood**	**Portugal**	**MT235721**	**MT235738**
	Halosphaeria torquata	BCC34303	Decayed driftwood	Taiwan	-	HQ111038
		BCC33480	Decayed driftwood	Taiwan	-	HQ111037
	Halosphaeria trifurcata	NTOU3773	-	-	-	KX686762

		PP2747	-	-	-	AF491277
	Halosphaeria tubulifera	BCC33511	Decayed driftwood	Denmark	-	HQ111026
		BCC33513	Decayed driftwood	Denmark	-	HQ111028
		BCC33512	Decayed driftwood	Wales	-	HQ111027
Kochiella	*Kochiella crispa*	BCC33504	Decayed driftwood	Denmark	-	HQ111018
		BCC33502	Decayed driftwood	Denmark	-	HQ111020
		BCC33507	Decayed driftwood	Denmark	-	HQ111019
Kohlmeyeriella	*Kohlmeyeriella tubulata*	PP1105	-	-	-	AF491265
		PP0989	-	-	-	AF491264
Naufragella	*Naufragella spinibarbata*	PP6886	Decayed driftwood	-	-	HQ111032
		BCC33508	Decayed driftwood	Wales	-	HQ111033
		BCC33482	Decayed driftwood	Taiwan	-	HQ111034
Ocostaspora	*Ocostaspora apilongissima*	CY3399	Decayed driftwood	Denmark	-	HQ111007
		NTOU4061	Driftwood	USA	-	KX686800
		LP32	Decayed driftwood	Denmark	-	HQ111006
Sigmoidea	*Sigmoidea parvula*	CBS 116644*	-	Germany	NR_160220	NG_064187
Outgroup	*Glomerulispora mangrovei*	NBRC 105264*	decayed intertidal wood at mangrove	Japan	NR_138418	NG_060628

BCC: BIOTEC Culture Collection, Thailand Biodiversity Center, National Center for Genetic Engineering and Biotechnology; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMG: Culture collection of Micael Gonçalves, housed at Department of Biology, University of Aveiro, Portugal; MUM: Culture collection hosted at Center for Biological Engineering of University of Minho, Portugal; NBRC: NITE Biological Resource Center of Japan; NTOU: National Taiwan Ocean University. Ex-type strains are marked with an asterisk. Sequences generated in this study are shown in bold.

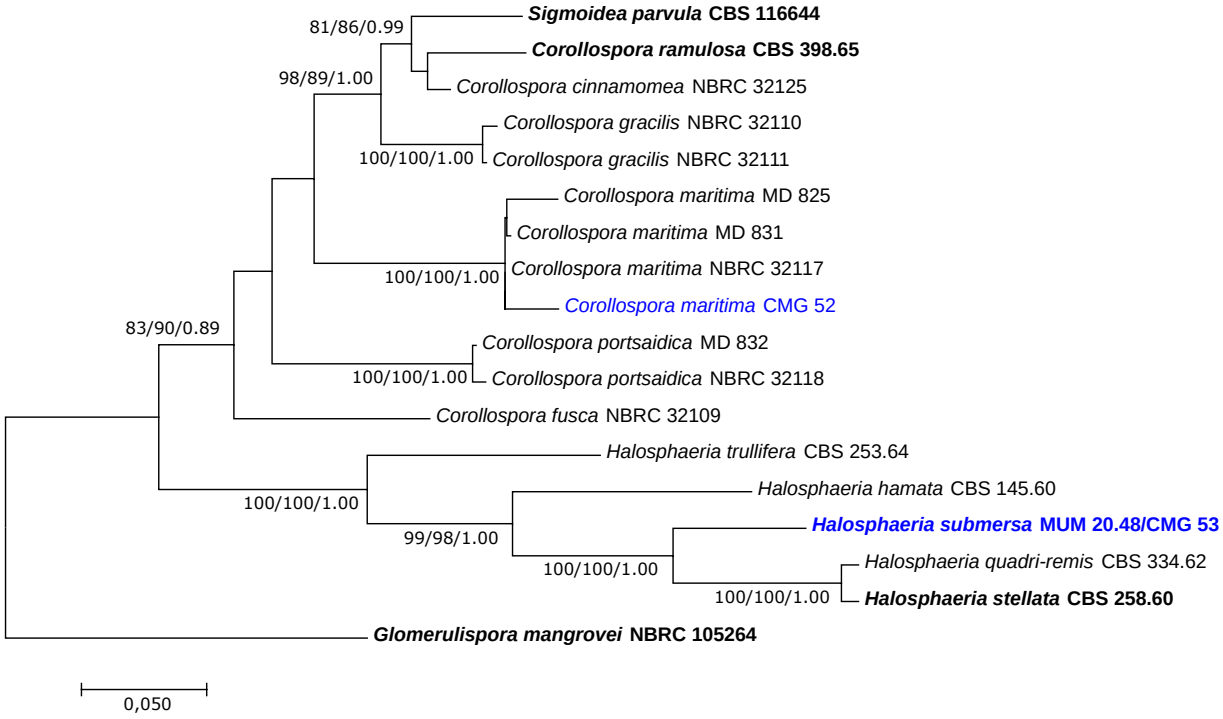


Fig. 1. Phylogenetic relationships of *Halosphaeriaceae* species based on combined LSU and ITS sequence data and inferred using the Maximum Likelihood method under the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Glomerulispora mangrovei* (NBRC 105264). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.

Regarding isolates CMG 54 and CMG 55, the closest matches for ITS sequence retrieved various hits, of which those with the highest sequence similarity belonged to unidentified isolates, such as Fungal sp. (GenBank accession no. KP757520; Identities 542/567 (96%), 10 gaps), Fungal sp. (GenBank accession no. KX013214; Identities 546/576 (95%), 8 gaps) and Fungal sp. (GenBank accession no. KP757525; Identities 541/567 (95%), 11 gaps). The closest match of an identified species was *Lulworthia* cf. *purpurea* (GenBank accession no. KT347218; Identities 545/575 (95%), 12 gaps) and *L.* cf. *purpurea* (GenBank accession no. KT347218; Identities 546/576 (95%), 13 gaps). Single-gene data set with ITS sequences were aligned separately with those of several related species of *Lulworthiaceae* family (Table 4) to access the species that are closest to our isolates before performing a multilocus

phylogenetic analysis. Additional molecular markers using LSU and SSU sequences were used to confirm the phylogenetic placement within the family *Lulworthiaceae*. The closest hits using the LSU sequence was *Zalerion xylestrix* (GenBank accession no. EU848592; Identities 1053/1101 (96%), no gaps), *Lulwoana uniseptata* (GenBank accession no. GU256630; Identities 1051/1101 (95%), no gaps), *L. uniseptata* (GenBank accession no. GU256628; Identities 1050/1102 (95%), 2 gaps) and for SSU sequence was *L.* cf. *purpurea* (GenBank accession no. AY879004; 1166/1170 (99%), 3 gaps), *L. uniseptata* (GenBank accession no. AY879034; Identities 1155/1170 (99%), 3 gaps) and *L. uniseptata* (GenBank accession no. AY879032; 1154/1170 (99%), 3 gaps). Thus, the alignment of the ITS, LSU + SSU and ITS + LSU + SSU contained 41, 26 and 60 sequences (including the outgroup), and there was a total of 663, 2885 and 2001 positions in the final dataset, respectively.

In all ML phylogenetic trees (Figs. 2, 3 and 4), the novel isolates CMG 54 and CMG 55 clustered in a clade that received high (100, 100 and 95%) bootstrap support with high PP values (1.00, 1.00 and 1.00) within the family *Lulworthiaceae* with a close relationship with the genus *Halazoon* (LSU + SSU), Figs. 3) and the species *Lulworthia* cf. *purpurea* and *Papulaspora halima* (ITS, ITS + LSU + SSU, Figs. 2 and 4).

BLASTn searches against the NCBI nucleotide database using the ITS sequences of CMG 64 and CMG 65 were *Z. maritima* (GenBank accession no. KT347217; Identities 553/553 (100%), no gaps), Ascomycete sp. (GenBank accession no. DQ124119; Identities 550/550 (100%), no gaps) and *Z. maritima* (GenBank accession no. AF169305; Identities 547/547(100%), no gaps). The LSU and the SSU genes were also sequenced to confirm the phylogenetic placement within the genus *Zalerion*. The highest similarities using the LSU sequence were *Z. xylestrix* (GenBank accession no. EU848592; Identities 1069/1072 (99%), 0 gaps), *L. uniseptata* (GenBank accession no. GU256630; Identities 1062/1072 (99%), no gaps) and *L. uniseptata* (GenBank accession no. GU256628; Identities 1061/1073 (99%), 2 gaps). Using the SSU sequence were *L. uniseptata* (GenBank accession no. AY879034; Identities 1068/1082 (99%), 6 gaps), *L. uniseptata* (GenBank accession no. AY879031; Identities 1068/1082 (99%), no gaps) and *L. uniseptata* (GenBank accession no. AY879032; Identities 1067/1082 (99%), 6 gaps). In all ML phylogenetic trees (Figs. 2, 3 and 4) the isolates CMG 64 and CMG 65 clustered in a different clade of *Z. maritima*.

Table 4. List of *Lulworthiaceae* isolates used in this study.

Genera	Species	Acession Number	Host/Substrate	Country	GenBank Acessions		
					ITS	SSU	LSU
Cumulospora	*Cumulospora marina*	MF46	-	Egypt	-	GU252136	GU252135
		GR53	-	Thailand	-	GU256625	GU256626
		JK4843	*Prosopis* sp.	Hawaii	-	AF195640	AF195641
		JK5393	*Rhizophora* sp.	Moorea	-	AF195638	AF195639
		JK5332A	-	-	-	AY879014	AY878971
Haloguignardia	*Haloguignardia irritans*	-	-	USA	-	AY566252	-
Halazoon	*Halazoon fuscus*	NBRC 105256*	Driftwood, dead rhizomes of *Phragmites communis*	-	-	GU252148	GU252147
	Halazoon melhae	PP2597	Decayed driftwood in the intertidal zone	Egypt	-	AY879030	AY878987
Hiogispora	*Hiogispora japonica*	JCM1753	on cortex of *Abies homolepis*	Japan	-	GU252140	GU252139
Hydea	*Hydea pygmea*	IT081	-	Thailand	-	GU256632	GU256633
		NBRC33069	decayed driftwood	Japan	-	GU252134	GU252133
		GR239	-	Thailand	-	-	GU256627
Lindra	*Lindra crassa*	ATCC 56663	-	Belize	-	AY878999	-
	Lindra obtusa	CBS 113030	-	Germany	-	AY879001	AY878959
		IFO31317*	-	Japan	-	AY879002	AY878960
		AFTOL-ID 5012	-	-	-	FJ176847	FJ176902
	Lindra thalassiae	JK4322	*Thalassia sp.*	Belize	-	AF195632	AF195633
		JK5090	detritus	USA	-	AF195634	AF195635
		AFTOL-ID 413	-	-	DQ491508	DQ470994	DQ470947
	Lindra marinera	JK5091A	-	USA	-	AY879000	AY878958
Lulwoana	*Lulwoana uniseptata*	CBS 280.54	-	-	MH857330	-	-

		CY3160	-	-	-	-	GU256628
		CBS 16760	-	-	-	AY879034	AY878991
		IFO32137*	Submerged wood	Japan	LC146746	AY879031	AY878988
		PP4032	-	-	-	AY879032	AY878989
		NBRC 32137	Submerged wood	Japan	LC146746	-	-
		LF848	Sediment	Atlantic Ocean:South Wales	-	KM096221	-
		LF834	Seawater	-	-	KM096218	-
Lulwoidea	*Lulwoidea lignoarenaria*	ATCC 64644	-	Denmark	-	AY879009	-
		AFTOL-ID 5013	-	-	-	FJ176848	FJ176903
		IFO32135	-	Japan	-	AY879010	AY878968
Lulworthia	*Lulworthia atlantica*	FCUL090707CF10*	Sea water	Portugal	KT347213	KT347199	JN886814
		FCUL210207SF10	Sea water	Portugal	-	KT347200	JN886833
		FCUL090707CF8	Sea water	Portugal	KT347212	-	JN886810
		FCUL061107CP4	Sea water	Portugal	KT347206	-	JN886826
		FCUL061107CP3	Sea water	Portugal	KT347208	KT347196	JN886825
		FCUL010407SP6	Sea water	Portugal	KT347209	KT347197	JN886836
		FCUL151007SP4	Sea water	Portugal	KT347210	KT347198	JN886842
		FCUL210208SP4	Sea water	Portugal	KT347205	KT347193	JN886843
	Lulworthia fucicola	ATCC 64288*	Submerged wood	Chile	-	AY879007	AY878965
	Lulworthia medusa	JK5581	*Spartina* sp.	USA: North Carolina	-	AF195636	AF195637
	Lulworthia opaca	CBS 21860	Submerged piles	USA	-	AY879003	AY878961
Lulworthiopsis	***Lulworthiopsis maritima***	**CMG 54**	**Submerged wood**	**Portugal**	**MT235722**	**MT235698**	**MT235739**
		MUM 20.50/CMG 55*	**Submerged wood**	**Portugal**	**MT235723**	**MT235699**	**MT235740**
	Lulworthiopsis purpurea comb. nov. (*Lulworthia cf. purpurea*)	CBS 219.60	Wood	Wales	-	AY879004	AY878962

		FCUL070108CF8	Sea water	Portugal	-	-	JN886818
		FCUL170907CF7	Sea water	Portugal	-	-	JN886815
		FCUL170907CP5	Sea water	Portugal	KT347219	KT347201	JN886824
		FCUL280207CF8	Sea water	Portugal	-	-	JN886807
		FCUL280207CF9	Sea water	Portugal	KT347218	KT347202	JN886808
		CMG 56	**Submerged wood**	**Portugal**	**MT235724**	**MT235700**	**MT235741**
		MUM 20.56/CMG 57*	**Submerged wood**	**Portugal**	**MT235725**	**MT235701**	**MT235742**
		CMG 58	**Submerged wood**	**Portugal**	**MT235726**	**MT235702**	**MT235743**
		CMG 59	**Submerged wood**	**Portugal**	**MT235727**	**MT235703**	**MT235744**
		CMG 60	**Submerged wood**	**Portugal**	**MT235728**	**MT235704**	**MT235745**
		CMG 61	**Submerged wood**	**Portugal**	**MT235729**	**MT235705**	**MT235746**
		CMG 62	**Submerged wood**	**Portugal**	**MT235730**	**MT235706**	**MT235747**
		CMG 63	**Submerged wood**	**Portugal**	**MT235731**	**MT235707**	**MT235748**
Marinomyces	*Marinomyces halima* comb. nov. (*Papulaspora halima*)	CBS 208.64*	Wood	USA	MH858421	-	MH870049
	Undescribed species	P20	*Posidonia oceanica*	Italy	KY465985	-	-
		P16	*Posidonia oceanica*	Italy	KY465982	-	KY486891
		P13	*Cymodocea nodosa*	Italy	KY465980	-	KY486888
		P5	*Cymodocea nodosa*	Italy	KY465975	-	KY486882
		P3	*Posidonia oceanica*	Italy	KY465973	-	KY486880
		PAV-M 1.2	Driftwood	Italy	KF915986	-	-
		PAV-M 1.167	Submerged wood	Italy	KF915995	-	-
		PAV-M 1.169	Driftwood	Italy	KF915992	-	-
		CMG 68	**Submerged wood**	**Portugal**	**MT235736**	**MT235712**	**MT235753**
		MUM 20.56/CMG 69	**Submerged wood**	**Portugal**	**MT235737**	**MT235713**	**MT235754**

Matsusporium	*Matsusporium tropicale*	IT061	-	Thailand	-	GU256631	-
		NBRC32499	-	Japan	-	GU252142	GU252141
Moleospora	*Moleospora maritima*	MF836*	decayed drift stems of *Phragmites australis*	Egypt	-	GU252138	GU252137
Moromyces	*Moromyces varius*	GR78	-	-	-	EU848593	EU848578
		IT152	-	-	-	EU848579	-
Orbimyces	*Orbimyces spectabilis*	G-cla3-SSU2_OTU-0-97_2	Rhizome-associated mycobiome	-	-	MF341571	-
Sammeyersia	*Sammeyersia grandispora*	JK 5168A*	Dead *Rhyzophora mangle*	Belize	-	AY879012	AY878969
		JK 5255[a]	Dead *Rhyzophora mangle*	Belize	-	AY879013	AY878970
		NTOU3843	Decayed mangrove wood	Taiwan	-	KY026045	-
		NTOU3841	Driftwood	Taiwan	-	KY026044	KY026048
		NTOU3847	Decayed mangrove wood	Taiwan	-	KY026046	KY026049
		NTOU3849	Decayed mangrove wood	Taiwan	-	KY026047	KY026050
Zalerion	*Zalerion maritima*	ATCC62580	driftwood log	USA	AF169305	-	-
		FCUL010407SP2	Seawater	Portugal	-	KT347204	-
		FCUL280207CP1	Seawater	Portugal	KT347216	KT347203	JN886806
		CMG 66	**Submerged wood**	**Portugal**	**MT235734**	**MT235710**	**MT235751**
		CMG 67	**Submerged wood**	**Portugal**	**MT235735**	**MT235711**	**MT235752**
	Zalerion pseudomaritima	**CMG 64**	**Submerged wood**	**Portugal**	**MT235732**	**MT235708**	**MT235749**
		MUM 20.49/CMG 65*	**Submerged wood**	**Portugal**	**MT235733**	**MT235709**	**MT235750**

AFTOL: Assembling the Fungal Tree of Life; ATCC American Type Culture Collection; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMG: Culture collection of Micael Gonçalves, housed at Department of Biology, University of Aveiro, Portugal; FCUL: Faculdade de Ciências da Universidade de Lisboa; IFO Institute for Fermentation, Osaka, Japan; MUM: Culture collection hosted at Center for Biological Engineering of University of Minho, Portugal; NBRC: NITE Biological Resource Center of Japan. Ex-type, holotype and neotype strains are marked with an asterisk. Sequences generated in this study are shown in bold.

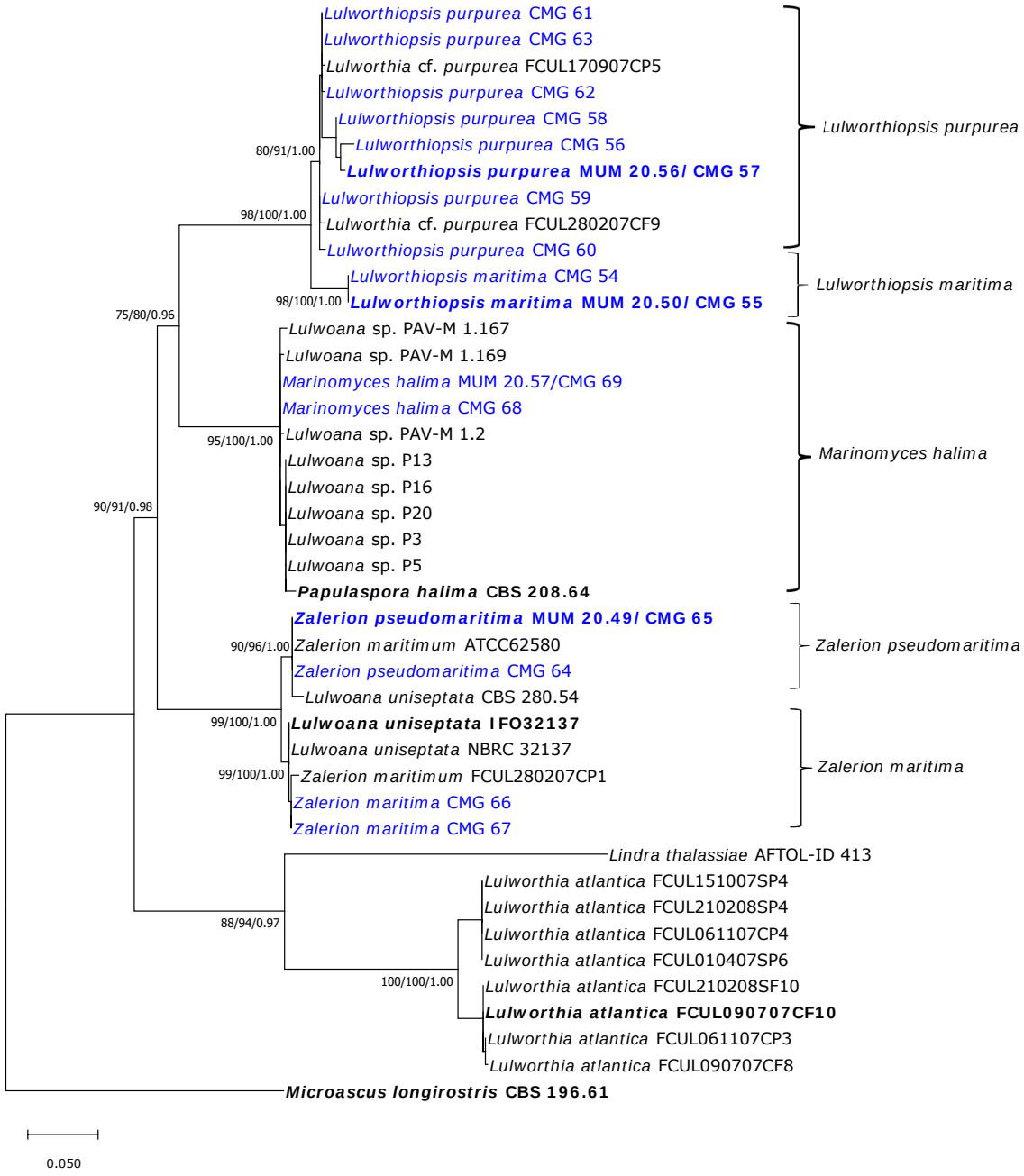
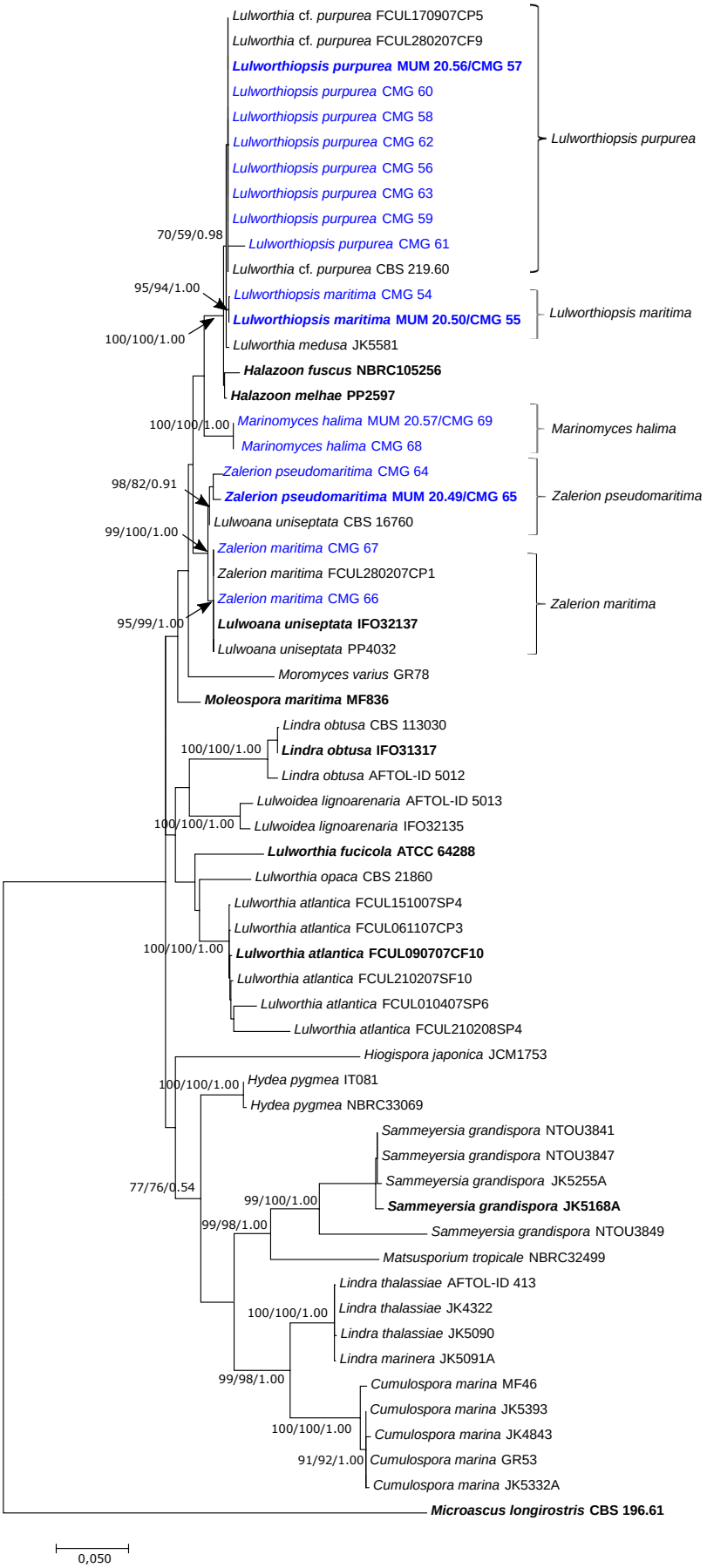


Fig. 2. Phylogenetic relationships of *Lulworthiaceae* species based on ITS sequence data and inferred using the Maximum Likelihood method under Tamura 3-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Microascus longirostris* (CBS 19661). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.


Lulworthia cf. purpurea FCUL170907CP5
Lulworthia cf. purpurea FCUL280207CF9
Lulworthiopsis purpurea MUM 20.56/CMG 57
Lulworthiopsis purpurea CMG 60
Lulworthiopsis purpurea CMG 58
Lulworthiopsis purpurea CMG 62
Lulworthiopsis purpurea CMG 56
Lulworthiopsis purpurea CMG 63
Lulworthiopsis purpurea CMG 59
70/59/0.98
Lulworthiopsis purpurea CMG 61
Lulworthia cf. purpurea CBS 219.60
Lulworthiopsis purpurea
95/94/1.00
Lulworthiopsis maritima CMG 54
Lulworthiopsis maritima MUM 20.50/CMG 55
100/100/1.00
Lulworthia medusa JK5581
Lulworthiopsis maritima
Halazoon fuscus NBRC105256
Halazoon melhae PP2597
100/100/1.00
Marinomyces halima MUM 20.57/CMG 69
Marinomyces halima CMG 68
Marinomyces halima
98/82/0.91
Zalerion pseudomaritima CMG 64
Zalerion pseudomaritima MUM 20.49/CMG 65
Lulwoana uniseptata CBS 16760
Zalerion pseudomaritima
99/100/1.00
Zalerion maritima CMG 67
Zalerion maritima FCUL280207CP1
Zalerion maritima CMG 66
95/99/1.00
Lulwoana uniseptata IFO32137
Lulwoana uniseptata PP4032
Zalerion maritima
Moromyces varius GR78
Moleospora maritima MF836
Lindra obtusa CBS 113030
100/100/1.00
Lindra obtusa IFO31317
Lindra obtusa AFTOL-ID 5012
Lulwoidea lignoarenaria AFTOL-ID 5013
100/100/1.00
Lulwoidea lignoarenaria IFO32135
Lulworthia fucicola ATCC 64288
Lulworthia opaca CBS 21860
Lulworthia atlantica FCUL151007SP4
Lulworthia atlantica FCUL061107CP3
100/100/1.00
Lulworthia atlantica FCUL090707CF10
Lulworthia atlantica FCUL210207SF10
Lulworthia atlantica FCUL010407SP6
Lulworthia atlantica FCUL210208SP4
Hiogispora japonica JCM1753
100/100/1.00
Hydea pygmea IT081
Hydea pygmea NBRC33069
Sammeyersia grandispora NTOU3841
Sammeyersia grandispora NTOU3847
77/76/0.54
Sammeyersia grandispora JK5255A
99/100/1.00
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99/98/1.00
Sammeyersia grandispora NTOU3849
Matsusporium tropicale NBRC32499
Lindra thalassiae AFTOL-ID 413
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Lindra thalassiae JK4322
Lindra thalassiae JK5090
Lindra marinera JK5091A
99/98/1.00
Cumulospora marina MF46
Cumulospora marina JK5393
100/100/1.00
Cumulospora marina JK4843
91/92/1.00
Cumulospora marina GR53
Cumulospora marina JK5332A
Microascus longirostris CBS 196.61
0,050

Fig. 3. Phylogenetic relationships of *Lulworthiaceae* species based on LSU and SSU sequence data and inferred using the Maximum Likelihood method under the General Time Reversible model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Microascus longirostris* (CBS 19661). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.

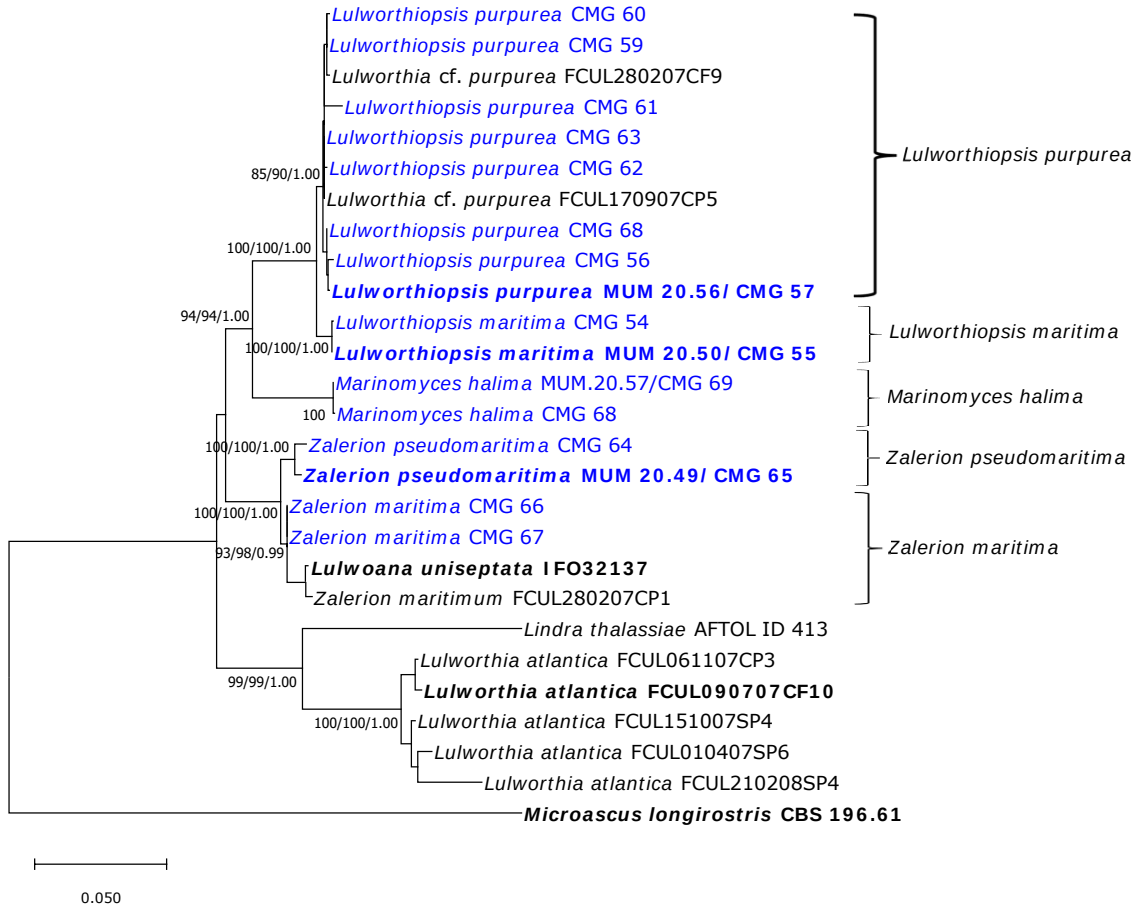

Fig. 4. Phylogenetic relationships of *Lulworthiaceae* species based on combined ITS, LSU and SSU sequence data and inferred using the Maximum Likelihood method under the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Microascus longirostris* (CBS 19661). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.

These three novel lineages in the genera *Lulworthiopsis* gen. nov., *Zalerion* and *Halosphaeria* are phylogenetically well delimited and are clearly distinct from the other closest species described so far and therefore are proposed here as a novel species.

Regarding isolates CMG 68 and 69 the closest matches for ITS sequence belonged to unidentified isolates, such as *Lulwoana* sp. (GenBank accession no. KF915986; Identities 514/514 (100%), no gaps), *Lulwoana* sp. (GenBank accession no. MN518387; Identities 512/512 (100%), no gaps). The closest match of an identified species was *Papulaspora halima* (GenBank accession no. MH858421; Identities 474/478 (99%, 2 gaps). In ITS phylogenetic tree (Fig. 2) our isolates, *Papulaspora halima* (CBS 208.64) and others unidentified species clustered together in a clade that received high (100%) bootstrap support with high PP values (1.00) in the family *Lulworthiaceae* with a closest relationship with genus *Zalerion* and *Lulworthiopsis*. Same relationships of our isolates and with the genus *Halazoon* were observed in phylogenetic tree of combined LSU and SSU (Fig. 3). In single SSU phylogenetic tree (Fig. S3) our isolates have a closest relationship with genus *Haloguignardia*. Large subunit ribosomal single tree is given in Fig. S4.

3.3. Taxonomy

Lulworthiopsis M. Gonçalves, A. Abreu & A. Alves, gen. nov.

MycoBank: MB 835053

Description. Sexual morph: mycelium consisting of septate, smooth hyphae, thick-walled, hyaline. Perithecia aggregated or solitary, globose to subglobose, black, immersed superficial, interior hyaline. Asexual morph unknown.

Etymology. The name reflects the morphological similarity in culture between this genus and *Lulworthia*.

Type species. *Lulworthiopsis purpurea* (I.M. Wilson) M. Gonçalves, A. Abreu & A. Alves, comb. nov.

Lulworthiopsis purpurea (I.M. Wilson) M. Gonçalves, A. Abreu & A. Alves, comb. nov.

Fig. 5

MycoBank: MB 835055

Basionym: *Halophiobolus purpureus* I.M. Wilson, Trans. Br. mycol. Soc. 39(4): 403 (1956).

= *Lulworthia purpurea* (I.M. Wilson) T.W. Johnson, Mycologia 50(2): 154 (1958).

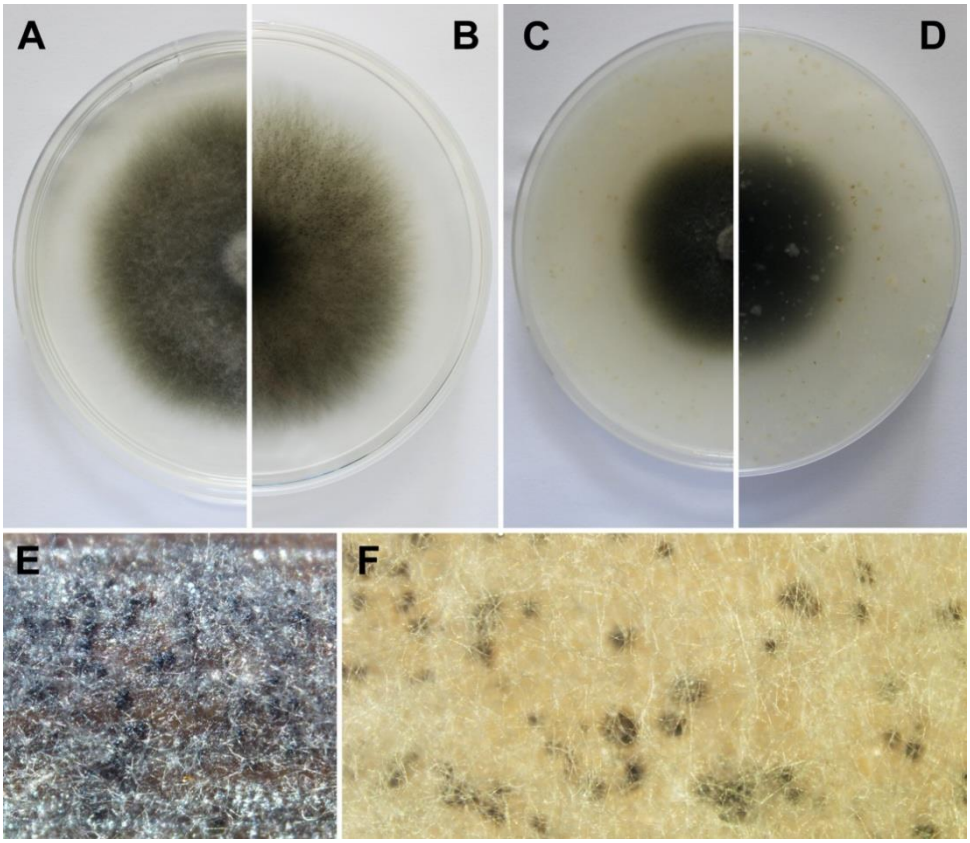

Fig. 5. *Lulworthiopsis purpurea* comb. nov. (MUM 20.56). A – B. Colony after 1 month at 25 ºC on PDA (obverse and reverse). C – D. Colony after 1 month at 25 ºC on CMA (obverse and reverse). E – F. Unknown structure after 1 month at 25 °C on pine needles and PDA.

Type. Great Britain, on submerged wood, 1952, I.M. Wilson, Herbarium IMI, (**holotype**, IMI 62908). **Portugal**, estuary Ria de Aveiro (40°37'48''N 8°43'58''W), isolated from submerged wood baits on May 2019, A. Abreu, deposited in the MUM Herbarium, (**epitype**

designated here: a dried culture sporulating, MUM-H 20.56; ex-epitype living culture, MUM 20.56 = CMG 57).

Description. Mycelium smooth, 1.5–2 µm wide hyphae. Hyphae thick-walled, smooth, septate, hyaline. Perithecia aggregated or solitary, globose to subglobose, black, immersed superficial, interior hyaline. Wilson (1956) described *Halophiobolus purpureus* as having perithecia scattered or grouped, immersed superficial, black, purple-brown under a microscope, 126-339 × 98-224 µm, perithecial wall membranous, surface colored, interior hyaline with 8-12-(20) µm, thickness. Neck central or not, dark-brown or black to gray peak. Asci elongated-davate containing eight ascosporous. Paraphvses not observed. Ascospores, curved or spiraled, hyaline, one-septa, multivacuolate, from both ends of the appendix; 190-257-(292) × 3-4 µm; appendages 10-16-(26) µm length. Habitat: a tree or rope, drowning at sea. Asexual morph unknown.

Culture characteristics. On 1-month old PDA and CMA plates, at 25 °C, colonies growing regular and above and a little immersed into agar with 60 and 40 mm in diameter, respectively. PDA obverse and reverse grey olivaceous to olivaceous black, with white tufts on the surface in PDA. CMA obverse and reverse olivaceous black. At 5 and 35 °C, there was no growth in any media tested.

Additional specimens examined. Portugal, Ria de Aveiro (Table 4), isolated from submerged wood. A. Abreu, living cultures CMG 56, CMG 58, CMG 59, CMG 60, CMG 61, CMG 62 and CMG 63.

Notes. *Lulworthiopsis purpurea* clustered in a distinct lineage in the family *Lulworthiaceae* with closest relationship with *Lulworthiopsis maritima*. Although the micromorphology of conidiogenous cells and conidia can be very similar in both species, they differ in terms of macromorphology and in quantity of conidia. As there are no living cultures that can be linked to the holotype, we have designated an epitype with live cultures linked to it, in order to stabilize the name and cultures are made available for future studies.

Lulworthiopsis maritima M. Gonçalves, A. Abreu & A. Alves, sp. nov. Fig. 6

MycoBank: MB 835054

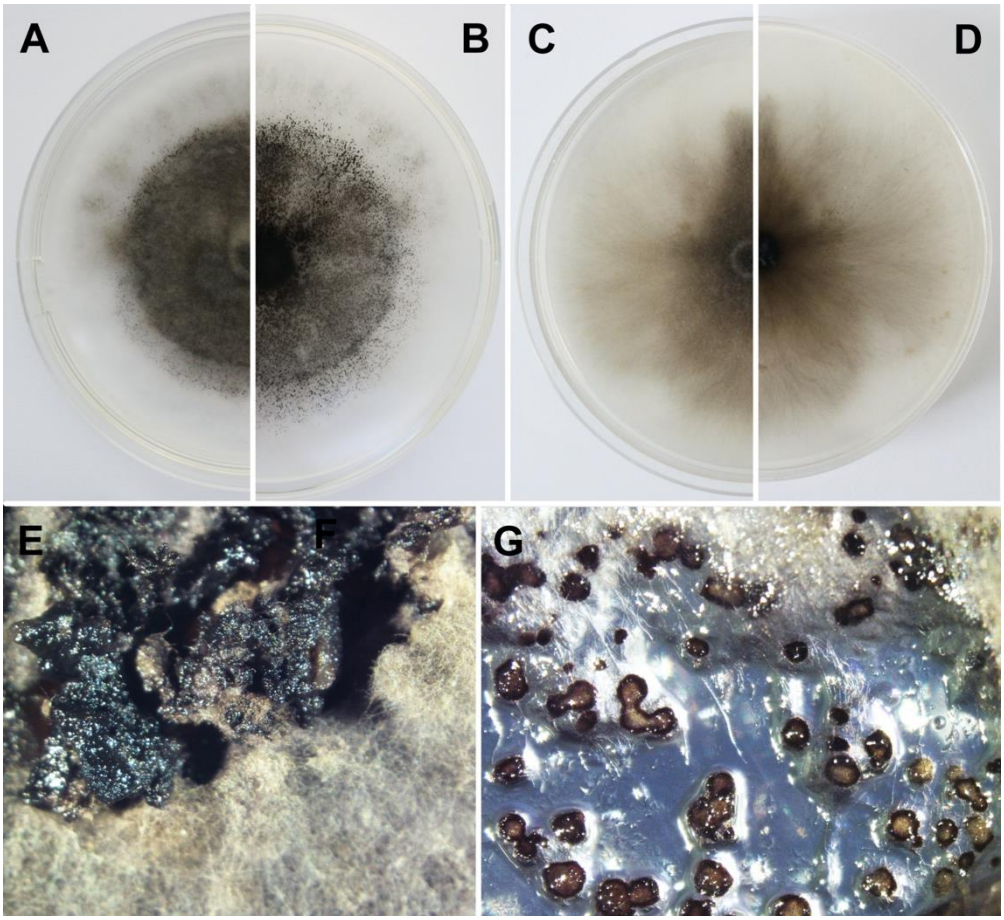

Fig. 6. *Lulworthiopsis maritima* (MUM 20.50). A – B. Colony after 1 month at 25 ºC on PDA (obverse and reverse). C – D. Colony after 1 month at 25 ºC on CMA (obverse and reverse). E – F. - Unknown structure after 1 month at 25 °C on pine needles and PDA.

Type. Portugal, estuary Ria de Aveiro (40°37'48''N 8°43'58''W), isolated from submerged wood baits on May 2019, A. Abreu, deposited in the MUM Herbarium, (**holotype**: a dried culture sporulating, MUM-H 20.50; ex-type living culture, MUM 20.50 = CMG 55).

Etymology. Referring to the environment where the species were collected, the sea (Latin: *Mare*).

Description. Mycelium smooth, 1.5–2 μm wide hyphae. Hyphae thick-walled, smooth, septate, hyaline. Perithecia aggregated, globose, black, immersed superficial, interior hyaline. Asci not observed. Asexual morph unknown.

Culture characteristics. On 1-month old PDA at 25 °C, colonies growing regular and above and a little immersed into agar with 72 mm in diameter. PDA obverse and reverse olivaceous black to greenish black. On 1-month old PDA at 25 °C, colonies growing irregular, above and a little immersed into agar with 80 mm in diameter. CMA obverse and reverse fuscous black to dark mouse grey. At 5 and 35 °C, there was no growth in any media tested.

Additional specimens examined. Portugal, Ria de Aveiro (Table 4), isolated from submerged wood. A. Abreu, living cultures CMG 54.

Notes: *Lulworthiopsis maritima* clustered in a distinct lineage in the genus *Lulworthiopsis* with closest relationship with *L. purpurea*. Macromorphologically, it resembles *L. purpurea* but they differ in shape and colour in culture media (Fig. 6). The phylogenetic trees demonstrate that *L. maritima* is phylogenetic distinct from *L. purpurea*. Also, they differ in 33, 6, 8 nucleotide positions in ITS, LSU and SSU, respectably.

Halosphaeria submersa M. Gonçalves, A. Abreu & A. Alves, sp. nov. Fig. 7

MycoBank: MB 835049

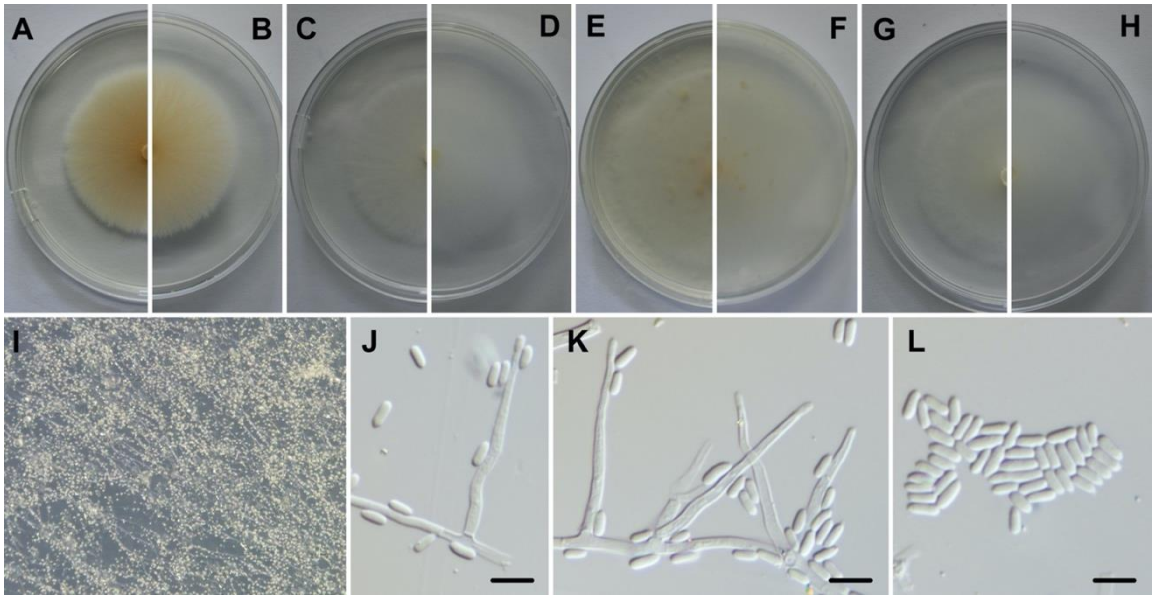

Fig. 7. *Halosphaeria submersa* (MUM 20.48). A – B. Colony after 15 days at 25 ºC on PDA (obverse and reverse). C – D. Colony after 15 days at 25 ºC on MEA (obverse and reverse). E – F. Colony after 15 days at 25 ºC on CMA (obverse and reverse). G – H. Colony after 15 days at 25 ºC on SNA (obverse and reverse). I. Colony texture in 15 days at 25 °C on SNA. J – K. Conidiophores. L. Conidia. Scale bar: 5 µm.

Type. Portugal, estuary Ria de Aveiro (40°37'48''N 8°43'58''W), isolated from submerged wood baits on May 2019, A. Abreu, deposited in the MUM Herbarium, (**holotype**: a dried culture sporulating, MUM-H 20.48; ex-type living culture, MUM 20.48 = CMG 53).

Etymology. Referring to the submerged habitat where the species were collected.

Description. Mycelium smooth, 1.5–2 µm wide hyphae. Hyphae thick-walled, smooth, septate, hyaline. Conidiophores reduced to conidiogenous cells erect, arising directly from vegetative hyphae, hyaline, smooth-walled, aseptate (mean ± SD = 28.9 ± 5.2 × 1.8 ± 0.3 µm, n = 30). Conidia aseptate, hyaline, smooth, cylindrical to narrowly ellipsoid (mean ± S.D. = 3.8 ± 0.5 × 1.5 ± 0.3 µm, n = 100). Chlamydospores not observed. Sexual morph unknown.

Culture characteristics. On 15 days old PDA, MEA, CMA and SNA at 25 °C, colonies growing regular and above and immersed into agar with 52, 52, 62 and 54 mm in diameter,

respectively. PDA obverse and reverse light-yellow ochre. MEA, CMA and SNA obverse and reverse yellowish white. At 5 °C, there was no growth in any media tested.

Marinomyces M. Gonçalves, A. Abreu & A. Alves, gen. nov.

MycoBank: MB 835051

Description. Mycelium consisting of septate, smooth hyphae, thick-walled, hyaline. Bulbils observed aggregated or solitary, globose to subglobose, irregular, black, submerged or superficial in agar. Propagules ("papulaspores") spherical, ovoid, ellipsoid or irregular, smooth to thick wall, hyaline, with long chains with tendency to detach each other when hold.

Etymology. Referring to the marine environment where it was found.

Type species. *Marinomyces halima* (Anastasiou) M. Gonçalves, A. Abreu & A. Alves, comb. nov.

Marinomyces halima (Anastasiou) M. Gonçalves, A. Abreu & A. Alves, comb. nov.
Fig. 8

MycoBank: MB 835052

Basionym. *Papulaspora halima* Anastasiou, Nova Hedwigia 6 (3-4): 266 (1963).

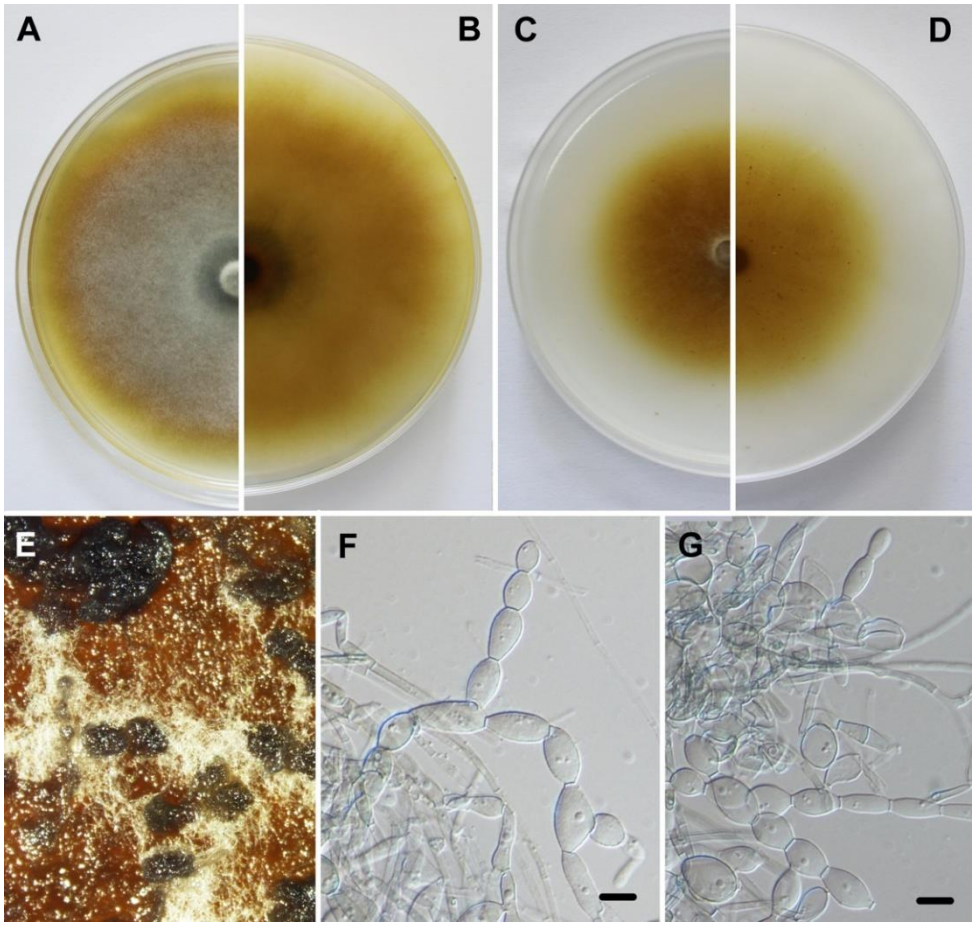

Fig. 8. *Marinomyces halima* (MUM 20.57). A – B. Colony after 15 days at 25 ºC on PDA (obverse and reverse). C – D. Colony after 15 days at 25 ºC on CMA (obverse and reverse). E. Bulbils. F – G. Propagules ("papulaspores"). Scale bar: G – 10 µm.

Type. USA, California, on *Tamarix aphylla* wood submerged in the southern marshes of the Salton Sea, July 20, 1961 (**holotype**: RSA23, ex-holotype living culture CBS 208.64).

Description. Hyphae septate, ramose, branched, smooth-walled, hyaline or lightly brown. Bulbils aggregated or solitary, globose to subglobose, irregular, black, submerged or superficial in agar. Cells spherical, ovoid, ellipsoid or irregular, smooth to thick wall, hyaline, with long chains detaching when hold (mean ± S.D. = 14.5 ± 2.3 × 9.7 ± 1.6 µm, n = 100). Anastaciou (1963) introduced *Papulaspora halima* as having mycelium branched, septate, hyaline, producing a brown pigment; septa with a refractive clamp, thickness, peripheral ring; bulbils hyaline to black in age, 35-500 µm diameter, of spherical cells 7.0-

15.7 × 6.1-12.2 µm. Bulbils formed from branching primordia in the serial mycelium and submerged in agar. Asexual morph unknown.

Culture characteristics. On 15 days old PDA and CMA plates, at 25 °C, colonies growing regular with 90 and 50 mm in diameter, respectively. PDA obverse ochreous in periphery with dirty white tufts on the surface getting darker in the center, producing a brown pigment, reverse ochreous to umber getting darker in the center. CMA obverse and reverse ochreous to umber. At 5 and 35 °C, there was no growth in any media tested.

Additional specimens examined. Portugal, estuary Ria de Aveiro (40°37'48''N 8°43'58''W), isolated from submerged wood baits on May 2019, A. Abreu, deposited in the MUM Herbarium, (a dried culture sporulating, MUM-H 20.57; living culture, MUM 20.57 = CMG 69). **Portugal**, Ria de Aveiro (Table 4), isolated from submerged wood. A. Abreu, living culture CMG 68.

Notes. *Marinomyces halima* clustered in a distinct lineage in the family *Lulworthiaceae* with closest relationship with the genus *Lulworthiopsis* and *Halazoon*. *Marinomyces halima* was doubtfully assigned to *Papulaspora* genus as *P. halima*. However, the "papulaspores" of this species do not show the characteristics sheath and central cells.

Zalerion R.T. Moore & Meyers, Can. J. Microbiol. 8: 408 (1962)
MycoBank: MB 10467

= *Lulwoana* Kohlm., Volkm.-Kohlm., J. Campb., Spatafora & Gräfenhan, Mycol. Res. 109(5): 562 (2005)

Description. The genus *Zalerion*, introduced by Moore and Meyers (1962) is characterized by having a normally black colony, felty, homogeneous, occasionally with white tufts in the center, without diffusible pigment and with dense conidial production.

Type species. *Zalerion maritima* (Lindner) Anastasiou, Can. J. Bot. 41: 1136 (1963)

Notes. The genus *Lulwoana* was introduced by Campbell et al. (2005) to accommodate the sexual stage of *Zalerion* (Moore and Meyers, 1962). With the ending of the dual nomenclature system for fungi it becomes necessary to decide which genus name to use. *Zalerion* is the oldest name and has priority over *Lulwoana*. Also, *Zalerion* includes more spcies names than the monotypic *Lulwoana* and is more commonly used. Thus, we recommend the use of genus *Zalerion* over *Lulwoana*.

Zalerion maritima (Lindner) Anastasiou, Can. J. Bot. 41: 1136 (1963) Fig. 9
MycoBank: MB 341143

Basionym: *Helicoma maritimum* Linder, Farlowia 1(3): 405 (1944)

= *Helicoma salinum* Linder, Farlowia 1(3): 406 (1944)

= *Zalerion nepura* R.T. Moore & Meyers, Can. J. Microbiol. 8: 413 (1962)

= *Zalerion eistla* R.T. Moore & Meyers, Can. J. Microbiol. 8: 413 (1962)

= *Zalerion xylestrix* R.T. Moore & Meyers, Can. J. Microbiol. 8: 414 (1962)

= *Zalerion raptor* R.T. Moore & Meyers, Can. J. Microbiol. 8: 415 (1962)

= *Lulwoana uniseptata* (Nakagiri) Kohlm., Volkm-Kohlm., J. Campb., Spatafora & Gräfenhan, Mycol. Res. 109(5): 562 (2005)

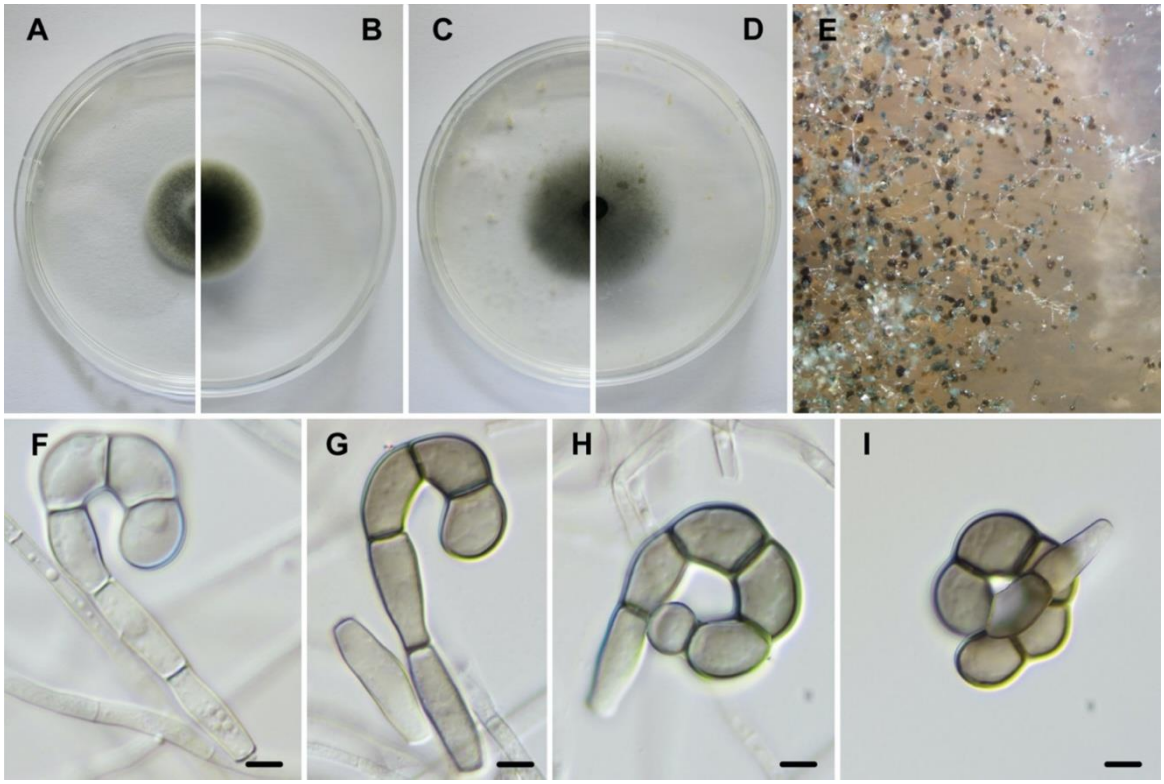

Fig. 9. *Zalerion maritima* (CMG 67). A – B. Colony after 15 days at 25 ºC on PDA (obverse and reverse). C – D. Colony after 15 days at 25 ºC on CMA (obverse and reverse). E. Colony texture in 15 days at 25 °C on CMA. F – I. Conidiogenous cells and conidia. Scale bar: 5 μm.

Type. Portugal, estuary Ria de Aveiro (40°37'48''N 8°43'58''W), isolated from submerged wood baits on May 2019, A. Abreu, deposited in the MUM Herbarium, (**neotype**: a dried culture sporulating, MUM-H (to be deposited); ex-neotype living culture, MUM (to be deposited) = CMG 67). **Japan**, Shizuoka Pref., Izu Pen., Shimoda, on submerged wood; 27 Aug. 1982, A. Nakagiri (**epitype**: TKB-F-5050, ex-epitype living culture IFO32137).

Description. *Zalerion maritima* clustered in a distinct lineage in the genus *Zalerion* with closest relationship with *Z. pseudomaritima*. Conidiogenous cells are hyaline to olivaceous (mean ± S.D. = 15.2 ± 2.0 × 4.8 ± 1.2 μm, n = 30) and the conidia are initially hyaline becoming olivaceous (mean ± S.D. = 54.8 ± 6.3 × 7.5 ± 0.7 μm, n = 100). According to Bills et al. (1999), conidia of *Z. maritima* have 17.6 – 29.6 μm × 4.8 – 8 μm, regular well-defined helices or tortuous. Sexual morph: *Lulwoana uniseptata* described by Campbell et al. (2005). Ascomata superficial or lightly immersed in wood, globose to subglobose, ostiolate, black

or dark brown. Neck cylindrical, straight or irregular. Peridium with two layers, being the external layer composed of polygonal or irregular dark cells and the internal layer is composed of flat or rhomboid hyaline cells. Hamathecium absent. Immature ascomata has pseudoparenchyma of polygonal, thin-walled and hyaline cells. Asci with eight ascospores, fusiform, curved, thin-walled, unitunicate, detaching early. Ascospores filiform, hyaline, one-septate, with a conical chamber at each end, releasing mucus from the tip.

Culture characteristics. On 15 days old PDA and CMA plates, at 25 °C, colonies growing regular with 26 and 38 mm in diameter, respectively. PDA and CMA obverse and reverse olivaceous black as observed by Bills et al. (1999). At 5 and 35 °C, there was no growth in any media tested.

Additional specimens examined. Portugal, Ria de Aveiro (Table 4), isolated from submerged wood. A. Abreu, living cultures CMG 66.

Notes. The genus *Zalerion* was introduced by Moore and Meyers (1962) to accommodate members of the Moniliaceae with spores formed by irregular spirals in three dimensions. As circumscribed by the authors the genus includes four species, *Z. nepura* (type species), *Z. eistla*, *Z. xylestrix*, and *Z. raptor*. At the time Moore and Meyers (1962) recognized affinity between these species and *Helicoma marinum* Lindner. Later, Anastasiou (1963) transferred *H. marinum* to *Zalerion* as *Z. maritima* Lindner (Anastasiou), regarded it as the type of *Zalerion* and synonymized all four *Zalerion* species under *Z. maritima*. Recently, Campbell et al. (2005) erected the genus *Lulwoana* with a single species *L. uniseptata* which was recognized as the sexual morph of *Z. maritima*. As dual nomenclature is no longer being used for fungi, we synonymize here *L. uniseptata* under *Z. maritima*.

Lindner (1944) listed three specimens in the original description of *H. maritimum*. None of these syntypes could be located at the Harvard University Herbarium and are presumably lost. Thus, a neotype is designated here to stabilize the species. In addition, as the neotype contains only the asexual morph a specimen containing the sexual morph is designated here as epitype.

Zalerion pseudomaritima M. Gonçalves, A. Abreu & A. Alves, sp. nov. Fig. 10

MycoBank: MB 835050

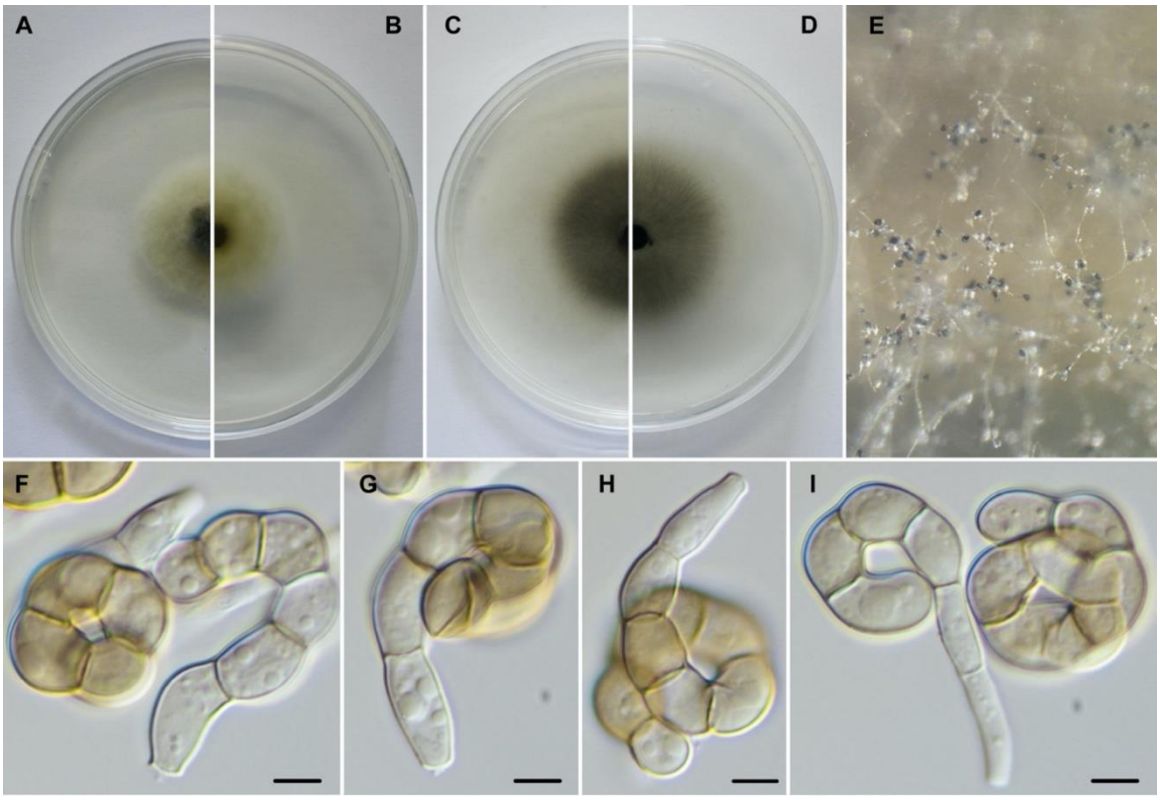

Fig. 10. *Zalerion pseudomaritima* (MUM 20.49). A – B. Colony after 15 days at 25 ºC on PDA (obverse and reverse). C – D. Colony after 15 days at 25 ºC on CMA (obverse and reverse). E. Colony texture in 15 days at 25 °C on CMA. F – I. Conidiogenous cells and conidia. Scale bar: 5 µm.

Type. Portugal, estuary Ria de Aveiro (40°37'48''N 8°43'58''W), isolated from submerged wood baits on May 2019, A. Abreu, deposited in the MUM Herbarium, (**holotype**: a dried culture sporulating, MUM-H 20.49; ex-type living culture, MUM 20.49 = CMG 65).

Etymology. Referring to the environment where the species were collected.

Description. Vegetative hyphae septate, branched, thick-walled, hyaline. Conidiogenous cells sub-cylindrical to narrowly clavate, thick-walled, hyaline to light golden brown (mean ± S.D. = 14.3 ± 2.3 × 4.6 ± 0.8 µm, n = 30). Conidia produced terminally or laterally on hyphae or short conidiophores. Conidial filament curved, sinuous or irregularly coiled, multi-septate, initially hyaline becoming golden brown. Groups of conidia appears

compactly intertwined, forming irregular shaped masses of cells (mean ± S.D. = 65.12 ± 11.5 × 7.6 ± 0.8 μm, n = 100). Sexual morph unknown.

Culture characteristics. On 15 days old PDA and CMA plates, at 25 °C, colonies growing regular with 34 and 70 mm in diameter, respectively. PDA obverse and reverse grey olivaceous to greenish olivaceous getting dark green in the center. CMA obverse and reverse darker dull green. At 5 and 35 °C, there was no growth in any media tested.

Additional specimens examined. Portugal, Ria de Aveiro (Table 4), isolated from submerged wood. A. Abreu, living cultures CMG 64.

Notes. *Zalerion pseudomaritima* clustered in a distinct lineage in the genus *Zalerion* with closest relationship with *Z. maritima*. Micromorphologically, it resembles *Z. maritima* but they differ in size, shape and colour of conidia (Fig. 12). Also, they differ in macromorphology. The single and combined phylogenetic trees demonstrates that *Z. pseudomaritima* is phylogenetic clearly distinct from *Z. maritima*. Also, they differ in 14, 10, 15 nucleotide positions in ITS, LSU and SSU, respectively.

4. Discussion

The submerged wood baits revealed a considerable diversity in the colonizing mycobiota, with 17 different species representing 10 fungal families recorded. Among the different taxa identified, all belong to the phylum Ascomycota with dominance of typically marine lignicolous fungi. However, we obtained other fungal species that are typically found in terrestrial environments which have also been commonly found in marine environments (Cantrell et al., 2006), including in association with submerged wood or driftwood (Garzoli et al., 2015, Gonçalves et al., 2019c) like species belonging to the genera *Penicillium*, *Cladosporium* and *Trichoderma*.

The most frequent species found in our collection belong to the family *Lulworthiaceae*, such as *Lulworthiopsis purpurea* (= *Lulworthia purpurea*), *L. maritima*, *Zalerion maritima* and *Z. pseudomaritima*. The dominance of *Lulworthiaceae* species associated to wood baits on temperate waters was already reported by Byrne and Jones (1974). This family comprises only marine ascomycetes (Kohlmeyer et al., 2000). Other marine species, which are

commonly associated to wood substrates found in other studies (e.g. *Ceriosporopsis halima, Cirrenalia macrocephala, Halosphaeria appendiculata, H. quadri-remis, Humicola alopallonella, Piricauda pelagica*) (Azevedo *et* al. 2010, 2011, Garzoli et al. 2015) were not detected in our study. One possible explanation for this is the geographic location. The studies made by Azevedo et al. (2010, 2011) was in two marinas, located in the western Portuguese coast (East Atlantic), while Garzoli et al. (2015) was in the Italian coastline (Mediterranean Sea). Comparing to the current study, that we only had one final sampling (after one year) and in one sampling location, Azevedo et al. (2010, 2011) collected the submerged baits periodically each eight to 10 weeks, for one year, encountering more and different species during ecological succession patterns. Other explanations could be the physical-chemical parameters to which the baits were submitted, like the salinity of seawater, temperature and the type of wood substrate. In fact, some studies reported that fungal community colonizing wood baits is dependent of the substrate. For example, Jones (2000), showed that *Lautisporopsis circumvestita* and *Cirrenalia macrocephala* show a significant preference for pine-wood substrates, while *Halosphaeria appendiculata* and *Marinospora longissima* prefer beechwood. Koch and Petersen (1997) investigated the occurrence of marine fungi associated with two types of wood substrate, such as oak (*Quercus* sp.) and larch (*Larix* sp.). Azevedo *et* al. (2010, 2011) used *Fagus sylvatica* and *Pinus pinaster* wood baits, while Byrne and Jones (1974) used also *F. sylvatica* and *P. sylvestris* wood, submerged in waters in the coast of England, and Grasso et al. (1990) used the same substrates in the Italian coast. All these studies evidenced a high predominance of *Lulworthiaceae* species, such *as Z. maritima* and *Lulworthia* sp., similar to what was observed in this study.

The family *Lulworthiaceae* was introduced by Kohlmeyer et al. (2000) in the order Lulworthiales and Maharachchikumbura et al. (2015) placed this order in the subclass Lulworthiomycetidae. Species of *Lulworthiaceae* are widely distributed in temperate and tropical oceans and are typically found growing on drift- or submerged wood or even in seaweeds (Kohlmeyer et al. 2000; Cannon and Kirk, 2007).

Recently, Azevedo et al. (2017) described a new *Lulworthia* species, namely *L. atlantica* isolated from *F. sylvatica* wood bait submerged in Cascais marina in Portugal. In the current study, two novel *Lulworthiaceae* species are described, namely *Z. pseudomaritima* and *Lulworthiopsis maritima*. *Z. pseudomaritima* is introduced in the genus *Zalerion* based on

phylogenetic and morphological analyses. The genus *Zalerion* was introduced by Moore and Meyers (1962). Currently, the genus comprises 13 species listed in Index Fungorum (2020) and MycoBank databases but there are eight species that do not have any DNA sequences available. However, there are some species named yet as *Zalerion* that have already been transferred to other genera such as *Z arboricola*, now as *Lophium arboricola*, *Z. longispiralis*, now as *Inesiosporium longispirale* and *Z. varia*, now as *Halenospora varia*, and others that are synonymous of *Z. maritima* such as *Z. eistla*, *Z. nepura*, *Z. raptor* and *Z. xylestrix*. New *Zalerion* species sharing similarities with *Z. maritima*, with differences in the conidia size, shape and color. The phylogenetic analysis provides also strong evidence that *Z. pseudomaritima* forms a sister clade to *Z. maritima* with high bootstrap support. Also based on morphological descriptions of *Zalerion* species available, these isolates may represent a new species, different of *Z. maritima* and the other described so far.

The taxonomic affiliation of some *Lulworthiaceae* species has been confusing with contrasting differences at phylogenetic and morphological level. An example of this is *Lulworthia purpurea*, that in our phylogenetic analysis and in other studies clustered separately from the genus *Lulworthia* (Campbell et al. 2005; Abdel-Wahab et al. 2010; Azevedo et al. 2017). At the morphological level, *Lulworthia purpurea* does not fit well in *Lulworthia sensu stricto* represented by *L. fucicola*, characterized by having ascospore dimensions of (66–) 77–110 (–121) × 4–6 mm and dark brown, globose ascomata with long necks (Campbell et al. 2005). Therefore, and in agreement with other authors (Koch et al. 2007; Jones et al. 2008; Abdel-Wahab et al. 2010) that reported this concern and anticipated the future establishment of a new genus, we propose a separate genus *Lulworthiopsis* to accommodate *Lulworthia* cf. *purpurea* as *L. purpurea*. Also, we identified *L. maritima* as a new species distinct from *L. purpurea*. Jones et al. (2015), already reported that *Lulworthia*-like species were referred to *Lulworthia sensu lato*, because many *Lulworthia* collections do not have morphological features documented and for practical purposes, unidentified species with ascospore lengths between 150–500 mm had been classified as *Lulworthia* sp. in the past (Kohlmeyer and Volkmann-Kohlmeyer, 1991).

For the other two isolates (CMG 68 and CMG 69), the closest match in BLASTn search of an identified species was *Papulaspora halima* CBS 208.64, isolated from wood in the USA (Anastasiou 1963). However, in our phylogenetic analysis this species clustered together with our isolates and other unidentified *Lulwoana* sp. isolates, receiving high (100%)

bootstrap support with high PP values (1.00) within the family *Lulworthiaceae*. Interestingly, all these unidentified *Lulwoana* sp. available in GenBank, were also isolated from marine substrates, such as seagrass species *Posidonia oceanica* and *Cymodocea nodosa* and drift- and submerged wood. These evidences also suggest that these isolates are well established in the family *Lulworthiaceae*. Actually, the taxonomic affiliation of *Papulaspora* species has been confusing leading to a new reclassification of many species, such as, *P. sepedonioides* as *Microthecium sepedonioides*, the type species that defined the genus *Papulaspora* (Marin-Felix and Guarro, 2018), *P. pulmonaria* as *Beverwykella pulmonaria* (Tubaki, 1975), *P. polyspora* as *Minimedusa polyspora* (Weresub and LeClair, 1971), *P. viridis* as *Trichoderma matsushimae* (Yamaguchi et al. 2012), among others. Moreover, Kohlmeyer and Kohlmeyer (2013) already reported that *P. halima* is doubtfully assigned to *Papulaspora*. Thus, based on these evidences and the results of our phylogenetic analyses we propose a new genus *Marinomyces* to accommodate *P. halima* as *M. halima*.

Other marine family well documented as colonizers of wood in the marine environment is *Halosphaeriaceae* (Abdel-Wahab and Nagahama, 2011; Pang *et* al. 2011; Pang and Jheng 2012; Chu *et* al. 2015), containing about 141 species, distributed through 86 genera (Jones *et* al. 2015). The genus *Halosphaeria* contains 20 species described to date and the majority of them appear to have some substrate preference, i.e. they can be found in association with decayed drift- or submerged wood. This study reports other *Halosphaeria* species isolated from submerged wood, namely *Halosphaeria submersa*. However, there were no ITS sequence data available for most species which are described as *Halosphaeria*, but there are LSU sequences for all *Halosphaeria* species and according to this single tree there is no doubts that we are dealing with a new species.

The present work supported the classification of some Lulworthiales taxa and provided more information about new species that are able to colonize submerged wood. Just as stated, marine lignicolous fungi have to deal with the several hostile conditions but they have to overcome to use the complex substrates on which they are developing (Garzoli et al. 2015). Therefore, lignicolous fungi possess a variety of enzymes that allow them to degrade the complex constituents of wood even in the marine environments. More studies focusing on production of interesting extracellular enzymes or secondary metabolites are needed to discover new valuable properties with a wide range of potential applications of these microorganisms.

A final consideration can be made about *Lulworthiaceae* and *Halosphaeriaceae* families, whose many species have a limited number of DNA sequences and nowadays it is very important for a correct species identification to perform a multi-gene phylogenetic and morphological analyses to resolve some taxonomic ambiguities.

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Supplementary material

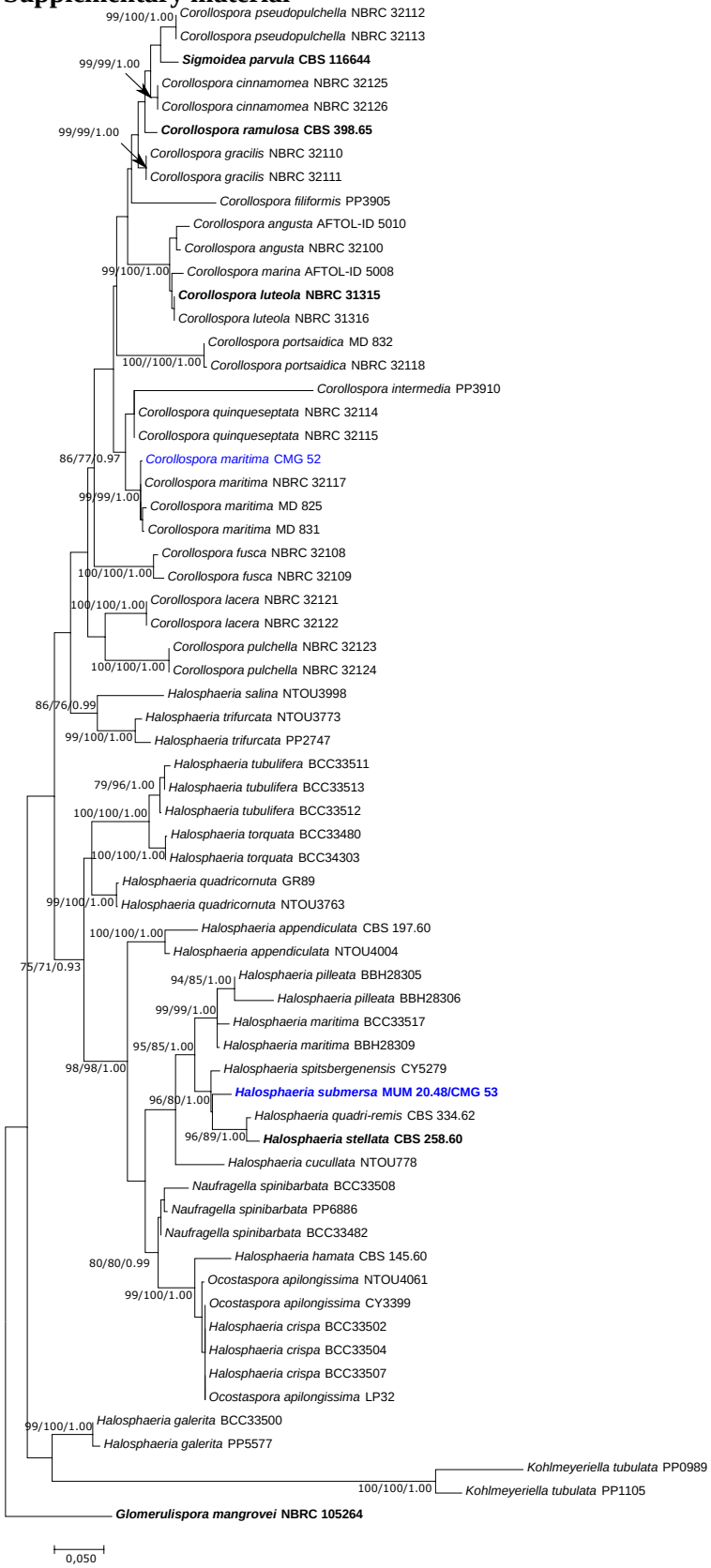


Figure S1. Phylogenetic relationships of *Halosphaeriaceae* species based on LSU sequence data and inferred using the Maximum Likelihood method under the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Glomerulispora mangrovei* (NBRC 105264). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.

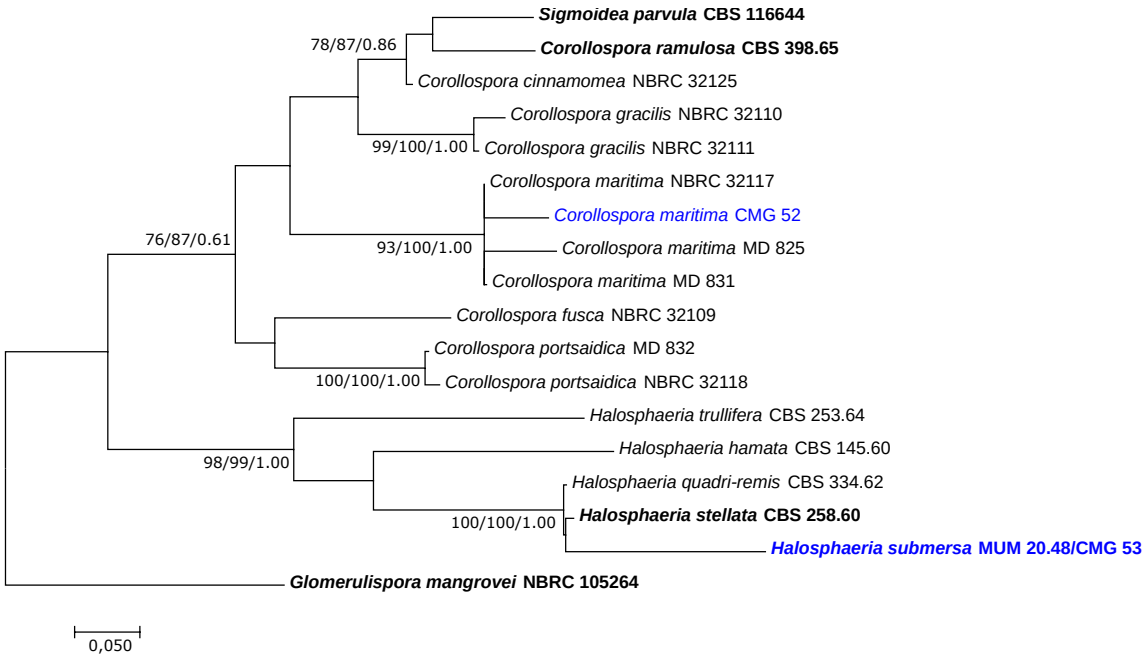
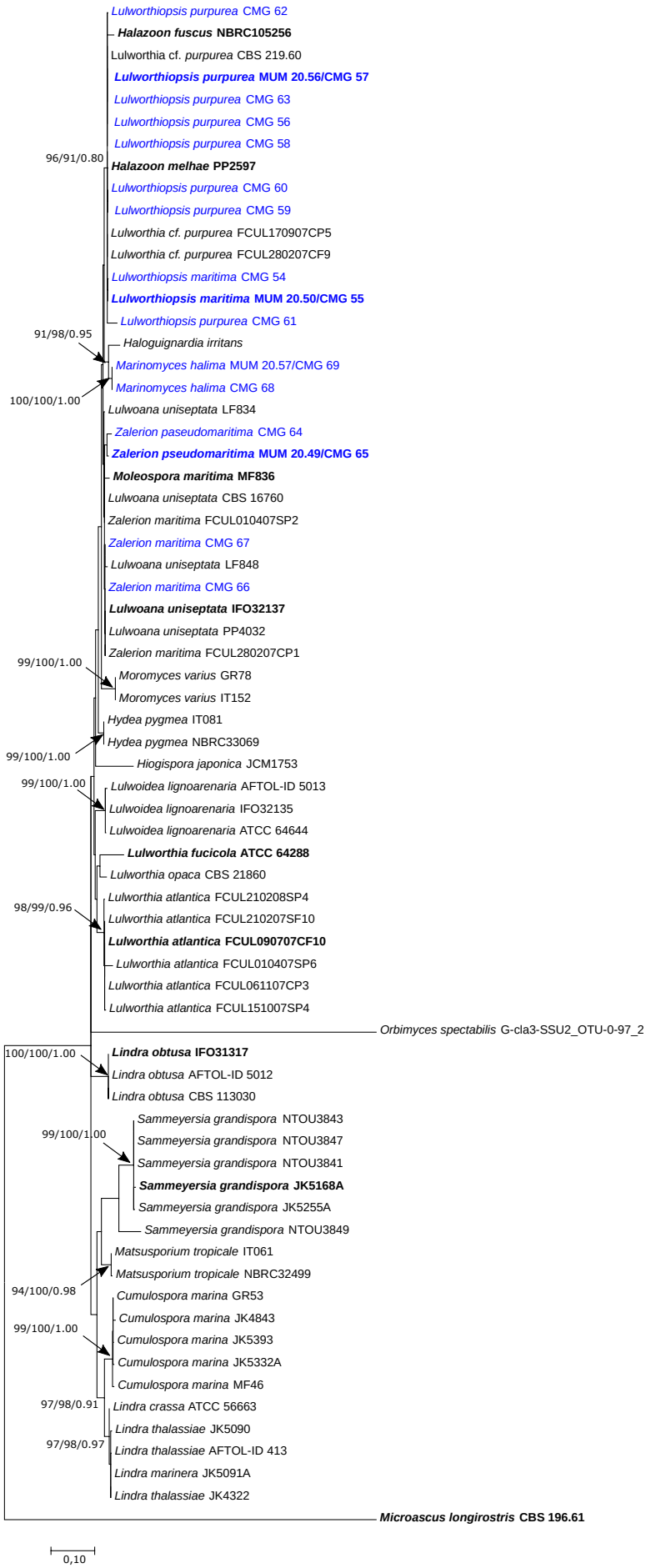


Figure S2. Phylogenetic relationships of *Halosphaeriaceae* species based on ITS sequence data and inferred using the Maximum Likelihod method under the Tamura 3-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Glomerulispora mangrovei* (NBRC 105264). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.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Lulworthia cf. purpurea CBS 219.60
Lulworthiopsis purpurea MUM 20.56/CMG 57
Lulworthiopsis purpurea CMG 63
Lulworthiopsis purpurea CMG 56
Lulworthiopsis purpurea CMG 58
96/91/0.80
Halazoon melhae PP2597
Lulworthiopsis purpurea CMG 60
Lulworthiopsis purpurea CMG 59
Lulworthia cf. purpurea FCUL170907CP5
Lulworthia cf. purpurea FCUL280207CF9
Lulworthiopsis maritima CMG 54
Lulworthiopsis maritima MUM 20.50/CMG 55
Lulworthiopsis purpurea CMG 61
91/98/0.95
Haloguignardia irritans
Marinomyces halima MUM 20.57/CMG 69
Marinomyces halima CMG 68
100/100/1.00
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Zalerion paseudomaritima CMG 64
Zalerion pseudomaritima MUM 20.49/CMG 65
Moleospora maritima MF836
Lulwoana uniseptata CBS 16760
Zalerion maritima FCUL010407SP2
Zalerion maritima CMG 67
Lulwoana uniseptata LF848
Zalerion maritima CMG 66
Lulwoana uniseptata IFO32137
Lulwoana uniseptata PP4032
Zalerion maritima FCUL280207CP1
99/100/1.00
Moromyces varius GR78
Moromyces varius IT152
Hydea pygmea IT081
Hydea pygmea NBRC33069
99/100/1.00
Hiogispora japonica JCM1753
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Lulwoidea lignoarenaria AFTOL-ID 5013
Lulwoidea lignoarenaria IFO32135
Lulwoidea lignoarenaria ATCC 64644
Lulworthia fucicola ATCC 64288
Lulworthia opaca CBS 21860
Lulworthia atlantica FCUL210208SP4
98/99/0.96
Lulworthia atlantica FCUL210207SF10
Lulworthia atlantica FCUL090707CF10
Lulworthia atlantica FCUL010407SP6
Lulworthia atlantica FCUL061107CP3
Lulworthia atlantica FCUL151007SP4
Orbimyces spectabilis G-cla3-SSU2_OTU-0-97_2
100/100/1.00
Lindra obtusa IFO31317
Lindra obtusa AFTOL-ID 5012
Lindra obtusa CBS 113030
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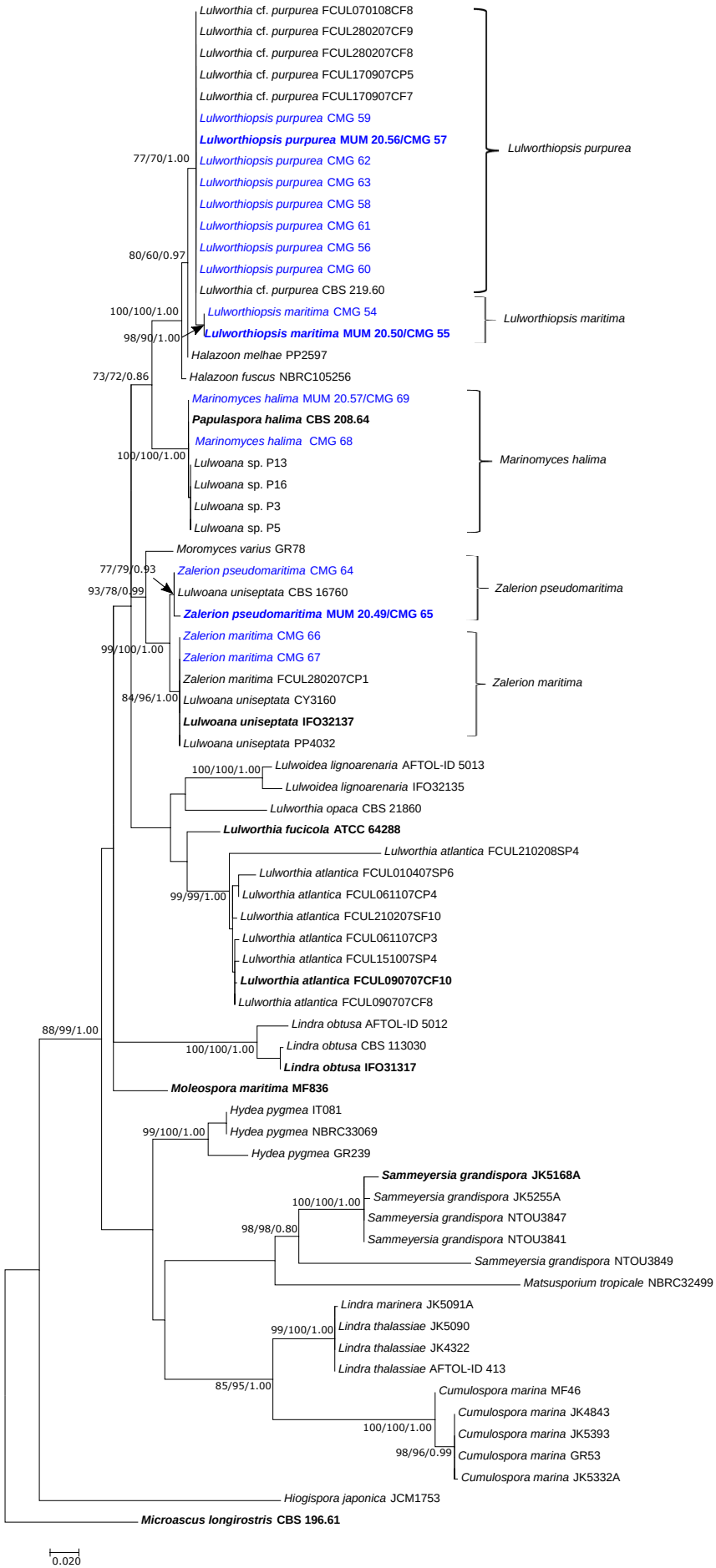
Sammeyersia grandispora NTOU3847
Sammeyersia grandispora NTOU3841
Sammeyersia grandispora JK5168A
Sammeyersia grandispora JK5255A
Sammeyersia grandispora NTOU3849
Matsusporium tropicale IT061
Matsusporium tropicale NBRC32499
94/100/0.98
Cumulospora marina GR53
Cumulospora marina JK4843
99/100/1.00
Cumulospora marina JK5393
Cumulospora marina JK5332A
Cumulospora marina MF46
97/98/0.91
Lindra crassa ATCC 56663
Lindra thalassiae JK5090
97/98/0.97
Lindra thalassiae AFTOL-ID 413
Lindra marinera JK5091A
Lindra thalassiae JK4322
Microascus longirostris CBS 196.61
0,10

Figure S3. Phylogenetic relationships of Lulworthiaceae species based on SSU sequence data and inferred using the Maximum Likelihod method under the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Microascus longirostris* (CBS 196.61). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue.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Lulworthia cf. purpurea FCUL170907CF7
Lulworthiopsis purpurea CMG 59
Lulworthiopsis purpurea MUM 20.56/CMG 57
77/70/1.00
Lulworthiopsis purpurea CMG 62
Lulworthiopsis purpurea CMG 63
Lulworthiopsis purpurea CMG 58
Lulworthiopsis purpurea CMG 61
80/60/0.97
Lulworthiopsis purpurea CMG 56
Lulworthiopsis purpurea CMG 60
Lulworthia cf. purpurea CBS 219.60
Lulworthiopsis purpurea
100/100/1.00
Lulworthiopsis maritima CMG 54
98/90/1.00
Lulworthiopsis maritima MUM 20.50/CMG 55
Lulworthiopsis maritima
Halazoon melhae PP2597
73/72/0.86
Halazoon fuscus NBRC105256
Marinomyces halima MUM 20.57/CMG 69
Papulaspora halima CBS 208.64
Marinomyces halima CMG 68
100/100/1.00
Lulwoana sp. P13
Lulwoana sp. P16
Lulwoana sp. P3
Lulwoana sp. P5
Marinomyces halima
Moromyces varius GR78
77/79/0.93
Zalerion pseudomaritima CMG 64
93/78/0.99
Lulwoana uniseptata CBS 16760
Zalerion pseudomaritima MUM 20.49/CMG 65
Zalerion pseudomaritima
Zalerion maritima CMG 66
99/100/1.00
Zalerion maritima CMG 67
Zalerion maritima FCUL280207CP1
84/96/1.00
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Lulwoana uniseptata IFO32137
Lulwoana uniseptata PP4032
Zalerion maritima
100/100/1.00
Lulwoidea lignoarenaria AFTOL-ID 5013
Lulwoidea lignoarenaria IFO32135
Lulworthia opaca CBS 21860
Lulworthia fucicola ATCC 64288
Lulworthia atlantica FCUL210208SP4
Lulworthia atlantica FCUL010407SP6
99/99/1.00
Lulworthia atlantica FCUL061107CP4
Lulworthia atlantica FCUL210207SF10
Lulworthia atlantica FCUL061107CP3
Lulworthia atlantica FCUL151007SP4
Lulworthia atlantica FCUL090707CF10
Lulworthia atlantica FCUL090707CF8
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Matsusporium tropicale NBRC32499
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Lindra thalassiae JK5090
Lindra thalassiae JK4322
Lindra thalassiae AFTOL-ID 413
85/95/1.00
Cumulospora marina MF46
Cumulospora marina JK4843
100/100/1.00
Cumulospora marina JK5393
98/96/0.99
Cumulospora marina GR53
Cumulospora marina JK5332A
Hiogispora japonica JCM1753
Microascus longirostris CBS 196.61
0.020

Figure S4. Phylogenetic relationships of *Lulworthiaceae* species based on LSU sequence data and inferred using the Maximum Likelihod method under the General Time Reversible model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Microascus longirostris* (CBS 196.61). Bootstrap values (> 70%) and posterior probabilities are shown at the nodes (ML/MP/BI). Ex-type strains are in bold and the sequence resulting from the current study are in blue

Chapter III

Screening of enzymatic and antibacterial activity of marine lignicolous fungi

Screening of enzymatic and antibacterial activity of marine lignicolous fungi

1. Introduction

Microorganisms such as fungi are able to colonize various substrates, develop on them and possibly create mutualistic or parasitic relationships (Tejesvi et al. 2007; Debbab and Proksch, 2012). Fungi produce a spectrum of degradative enzymes crucial for colonization of the host, as well as in for the degradation of organic matter to obtain nutrients (Hankin and Anagnostakis, 1975). Most of the studies related to mycobiotechnology come from terrestrial fungi. However, Kobayashi and Ishibashi (1993) argue that the group of marine fungi could produce secondary metabolites different from terrestrial ones, also with biotechnological applications. This difference can be explained by the evolutionary development of specific secondary metabolic pathways driven by environmental conditions in the marine environment, such as temperature, nutrient availability, competition, salinity and symbiotic mode of life with marine plants and animals (LiBerra and Lindequist, 1995; Raghukumar, 2017a). Even in small concentrations, the secondary metabolites produced by these microorganisms represent a source of drugs for the therapeutic industry (Balaji et al. 2013). The truth is that in recent decades, studies looking for and discovering compounds of interest in marine fungi have increased (Hasan et al. 2015; Raghukumar, 2017a). Between 2000 and 2010, about seven hundred and ninety compounds produced by fungi that inhabit the marine environment were reported (Duarte et al. 2012). Currently, it is known that these compounds can have antifungal (Xu et al. 2015; Ding et al. 2019), antiviral (Rowley et al. 2003; Yasuhara-Bell and Lu, 2010), antibacterial (Samuel et al. 2011; Xu et al. 2015), anti-inflammatory (Kim et al. 2013; Xu et al. 2019), cytotoxic (Belofsky et al. 1998; Jans et al. 2017) and anti-cancer (Deshmukh et al. 2018; Ramos et al. 2016) properties.

Marine fungi are mostly found on lignocellulosic substrates containing cellulose, lignin, hemicellulose and pectin in their composition, such as in the decomposition of vascular plants, algae and wood (Rohrmann and Molitoris, 1992). Wood substrates are considered allochthonous materials from terrestrial plants, which end up reaching the marine environment in several ways: dead parts of trees or woody plants (driftwood) that end up floating in estuaries or sea coasts; constructions of anthropological origin, such as boats, pillars and docks; lignocellulose ropes, often used to dock boats and other nautical objects (Raghukumar, 2017a). Something that has been observed in several studies is the fact that

the type of wood influences the diversity of marine fungi associated to it, like for example, *Lautisporopsis circumvestita* and *Cirrenalia macrocephala* show a significant preference for pine-wood substrates, while *Halosphaeria appendiculata* and *Marinospora longissima* prefer beechwood (Jones, 2000). Also, Rämä et al. 2016 used high-throughput sequencing to demonstrate that fungal communities in conifer logs differ from deciduous ones. Enzymatic degradation of wood is one of the most important aspects in the growth of lignicolous marine fungi (Raghukumar, 2017a). This interaction with the substrate is a source of numerous lignocellulolytic enzymes with biotechnological potential, such as cellulases, hemicellulases and lignin-degrading enzymes (LDEs) (Motta et al. 2011; Sajith et al. 2016). Lignicolous mangrove fungi such as *Halorosellinia oceanica*, *Lignincola laevis* and *Trematosphaeria mangrovei* are capable of producing extracellular cellulases, namely endoglucanase, cellobiohydrolase and β-glucosidase (Pointing and Hyde, 2000). *Lophiostoma mangrovei* and *Hypoxylon oceanicum* are able to produce hemicellulases such as xylanase (Raghukumar et al. 1994), an enzyme useful in wood processing in the paper industry (Liu and Kokare, 2017). Other lignicolous fungi isolated from mangrove debris, such as *Lulworthia* species are capable of producing high levels of laccase (Raghukumar et al. 2008), useful for lignin mineralization to CO_2 (Sutherland et al. 1982). The exploitation of the extracellular activity of these fungi has contributed to the development of products used in various industrial sectors (paper, alcohol distillation, textiles) as well as in the discovery and use of bioremediation techniques (Damare et al. 2012; Garzoli et al. 2015; Raghukumar, 2017b).

This study aimed to perform a screening regarding the enzymatic and antibacterial activities of lignicolous marine fungi isolated from the estuary Ria de Aveiro (Portugal).

2. Materials and Methods

2.1. *Fungi isolates used in the screenings of enzymatic and antibacterial activity*

The fungi isolates used in this study were: *Lulworthiopsis maritima* (MUM 20.50/CMG 55), *L. purpurea* (MUM 20.56/CMG 57), *Halosphaeria submersa* (MUM 20.48/CMG 53), *Marinomyces halima* (CMG 69), *Sedecimiella taiwanensis* (CMG 51), *Zalerion pseudomaritima* (MUM 20.49/CMG 65) and *Z. maritima* (CMG 67). These isolates were all isolated from submerged driftwood during 1 year in the estuary Ria de Aveiro, Portugal (Chapter 2). They were cryopreserved, in 15% glycerol at -80ºC, and during the essays were maintained in Potato Dextrose Agar (PDA), prior to testing.

2.2 *Detection of extracellular activity*

For the enzymatic screening, ten different types of culture media were used in order to detect extracellular activity of the following enzymes: amylase, caseinase, cellulase, gelatinase, laccase, pectin lyase, pectinase, chitinase, urease and xylanase. There were two conditions of each enzyme: with and without 3% sea salts (Sigma-Aldrich, Darmstadt, Germany). All agar media were inoculated with a 7 mm diameter agar plug, previously removed from a growing culture (14 days old) and kept at 25 ºC. The tests were only started when there was a micellar diameter of 3 cm.

a) Amylase activity

Detection using the following culture medium (g/litre): 10g of peptone, 5g of yeast extract, 5g of sodium chloride (NaCl), 2g of starch and 15g of bacto-agar. For the revelation of enzyme activity, plates were flooded with Lugol solution.

Positive activity: visible yeallow or brown halo around the mycelium (Hankin and Anagnostakis, 1975).

b) Caseinase activity

Detection using the following composition (g/litre): 10g of skim milk, 5g of malt extract and 15g of bacto-agar.

Positive activity: visible clear or transparent halo around the mycelium (Kaur and Padmaja, 2009).

c) Cellulases and xylanases activities

Detection using the following culture medium (g/litre): 3g of sodium nitrate ($NaNO_3$), 1g of monopotassium phosphate (KH_2PO_4), 0.5g of magnesium sulfate ($MgSO_4$), 1g of yeast extract, 5g of carboxymethylcellulose or xylan (for cellulase and xylanase media, respectively) and 15g of bacto-agar. For the revelation of these two enzymatic activities, plates were flooded with Congo Red (1mg/mL; 15 min) and distained with 1M NaCl.

Positive activity: visible yellow halo around the mycelium (St Leger *et* al. 1997).

d) *Gelatinase activity*

Detection using the following composition (g/litre): 10g of gelatin, 5g of malt extract and 15g of bacto-agar.

Positive activity: visible clear or transparent halo around the mycelium (Kumar *et* al. 2009).

e) Laccase activity

Detection using the following culture medium (g/litre): 10g of tannic acid, 15g of malt extract and 20g of bacto-agar. The tannic acid solution and malt extract agar were autoclaved separately and mixed together after.

Positive activity: observation of a brownish color around the mycelium (Rigling, 1995).

f) Pectinase and pectin lyases activity

Detection using the following culture medium (g/litre): 0.3 of sodium nitrate ($NaNO_3$), 0.1g of monopotassium phosphate (KH_2PO_4), 0.5g of magnesium sulfate ($MgSO_4$), 1g of yeast extract, 5g of pectin and 15g of bacto-agar. For the pectinase testing media, pH was set to 5.0. As for the pectin lyase testing media the pH was set to 7.0. For the revelation of these two enzymatic activities, plates were flooded with Lugol solution.

Positive activity: visible yellow/brown (for pectinase) and transparent halo (for pectin lyase) around the mycelium (Hankin & Anagnostakis, 1975; St Leger *et* al. 1997).

g) Chitinase activity

Detection using the following culture medium (g/litre): 4.5g of colloidal chitin, 0.3g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 3g of ammonium sulfate (NH_4SO_4), 2g of monopotassium phosphate (KH_2PO_4), 1g of citric acid monohydrate ($C_6H_8O_7.H_2O$), 15g of bacto-agar and 0.15g of bromocresol purple ($C_{21}H_{16}Br_2O_5S$). The pH was set to 4.7.

Positive activity: visible purple halo around the mycelium (Fenice *et* al. 1998).

The preparation of colloidal chitin was adapted from Agrawal and Kotasthane (2012). Briefly, 1g of chitin was dissolved in 20ml of concentrated HCl; left on a magnetic stirrer overnight at 4˚C, at high speed; 500ml of ice-cold 96% ethanol was added and stirred for 2h; centrifuged at 3000 rpm for 10 minutes at 4ºC; the precipitate was washed with NaOH repeatedly, until the pH of the solution was 7; colloidal chitin was finally dried with absorbent filter paper or heater.

h) Urease activity

Detection using the following culture medium (g/litre): 1g of peptone, 1g of D-glucose, 5g of sodium chloride (NaCl), 2g of monopotassium phosphate (KH_2PO_4), 15g of bacto-agar, 500 ml of urea at 40% and 0.012 of phenol red. Urea was sterilized separately by filtration (0.2µm pore) and added to the medium after autoclaving it.

Positive activity: visible color change to red or dark pink (Hankin & Anagnostakis, 1975).

2.3 Detection of antibacterial activity

For the antibacterial screening, six different bacteria were used in order to detect susceptibility to compounds produced by our fungal isolates: three gram-positive, namely *Staphylococcus aureus* (ATCC 6538), *Kocuria rhizophila* (ATCC 9341) and *Enterococcus faecalis* (ATCC 29212) and three gram-negative species, *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (P9.5) and *Pseudomonas aeruginosa* (4P6.2). Plugs with fungal mycelium were inoculated, in 90 mm plates with PDA, at the right end of the plate. Just like in enzymatic screening, there were two conditions: PDA plates enriched with and without 3% sea salts. The inoculated plates were incubated at 25ºC, until the mycelium reached more and less to the middle of the plate. To obtain pure cultures of each bacterial isolate, streaks were carried out using Plant Counting Agar (PCA), incubated overnight at 37ºC. Then, cell suspensions were prepared using the pure bacterial colonies and adjusted to turbidity of 0.5 McFarland. The streaking of the six bacteria were made on the mycelial plates and incubated overnight at 37ºC.

3. Results

a) Screening of enzymatic activity

The results regarding the extracellular activity of fungal isolates are shown in Table 1.

Table 1. Fungal isolates used in the detection of extracellular activity.

Species	Culture No.	Time* (days)	Amylases		Cellulases		Xylanases		Pectinanes		Pectin lyases		Caseinases		Ureases		Gelatinases		Laccases		Chitinases	
			w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt	w/ salt	w/o salt
Lulworthiopsis maritima	MUM 20.50 / CMG 55	16	-	-	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-
Lulworthiopsis purpurea	MUM 20.56 / CMG 57	10	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Halosphaeria submersa	MUM 20.48 / CMG 53	6	-	-	-	+	-	-	+	+	-	-	+	+	+	+	-	+	-	-	+	+
Marinomyces halima	MUM 20.57 /CMG 69	7	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-
Sedecimiella taiwanensis	CMG 51	6	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-
Zalerion pseudomaritima	MUM 20.49 / CMG 65	6	-	-	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-
Zalerion maritima	/CMG 67	10	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-

CMG: Culture collection of Micael Gonçalves, housed at Department of Biology, University of Aveiro, Portugal; MUM: Culture collection hosted at Center for Biological Engineering of University of Minho, Portugal. *: Time until the mycelium reach about 3 cm in diameter.

Regarding the results of the enzymatic activity, it is necessary to highlight that for some enzymes, the fungal isolates selected showed different enzymatic activity in the presence or absence of sea salts.

The majority of fungal isolates showed cellulolytic and pectinolytic activity. Contrarily to the other enzymatic activities, laccase activity was not detected in any of the isolates tested. Chitinase activity was only detected in *Halosphaeria submersa* in both conditions and gelatinase activity was only detected in media without sea salt in *H. submersa* and *Sedecimiella taiwanensis*.

Regarding amylase and xylanases activity, these were detected only in *Marinomyces halima* and *Zalerion maritima* in both conditions. *S. taiwanensis* also demonstrated to have amylolytic activity without salt.

Within *Zalerion* and *Lulworthiopsis* species there was a contrast in production of enzymatic activity. For example, *Z. pseudomaritima* produced amylase, while *Z. maritima* did not, or *L. maritima* and *L. purpurea* in their pectin lyases activity.

Caseinase and urease activity were only observed in *H. submersa* and *S. taiwanensis* in both conditions.

b) Screening of antibacterial activity

The results regarding the antibacterial activity of the fungal isolates are shown in Table 2.

Table 2. Fungal isolates used in the detection of antibacterial activity.

Species	Strain	Time * (d)	Condition	Length (mm)	Gram Positive Bacteria			Gram Negative Bacteria		
					Staphylococcus aureus ATCC 6538	*Kocuria rhizophila* ATCC 9341	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* ATCC 25922	*Klebsiella pneumoniae* (P9.5)	*Pseudomonas aeruginosa* (4P6.2)
Lulworthiopsis maritima	MUM 20.50/ CMG 55	33	**w/ salt**	19 ± 0.1	-	-	-	-	-	-
			w/o salt	7 ± 0.1	-	-	-	-	-	-
Halosphaeria submersa	MUM 20.48 / CMG 53	16	**w/ salt**	24 ± 0.1	-	-	-	-	-	-
			w/o salt	26 ± 0.1	-	-	-	-	-	-
Marinomyces halima	MUM 20.57 CMG 69	14	**w/ salt**	23 ± 0.1	+	-	-	+	-	-
			w/o salt	35 ± 0.1	+	+	+	+	-	+
Lulworthiopsis purpurea	MUM 20.56 / CMG 57	16	**w/ salt**	38 ± 0.1	-	-	-	-	+	-
			w/o salt	14 ± 0.1	+	-	+	-	+	-
Sedecimiella taiwanensis	CMG 51	8	**w/ salt**	34 ± 0.1	-	-	-	-	-	-
			w/o salt	15 ± 0.1	-	-	-	-	+	-
Zalerion maritima	CMG 67	27	**w/ salt**	21 ± 0.1	+	+	-	-	-	-
			w/o salt	12 ± 0.1	-	-	-	-	-	-
Zalerion pseudomaritima	MUM 20.49 / CMG 65	27	**w/ salt**	31 ± 0.1	+	+	+	-	-	-
			w/o salt	16 ± 0.1	+	+	+	-	-	-

CMG: Culture collection of Micael Gonçalves, housed at Department of Biology, University of Aveiro, Portugal; MUM: Culture collection hosted at Center for Biological Engineering of University of Minho, Portugal. *: Time until the mycelium reach half of the plate.

Regarding the results of antibacterial activity, the selected isolates showed better results against Gram-positive bacteria, like *Z. maritima, Z. pseudomaritima, M. halima* and *L. purpurea*. Also, *L. purpurea*, *M. halima* and *S. taiwanensis* showed antibacterial activity against the multi-resistant Gram-negative bacteria. For example, *L. purpurea* and *S. taiwanensis* showed activity against *K. pneumoniae* and *M. halima* against *E. coli* and *P. aeruginosa*. However, the isolates showed different antibacterial activity when cultivated in media with and without sea salts. *Zalerion maritima* showed antibacterial activity only in media with salt, against *S. aureus* and *K. rhizophila*, while *L. purpurea* in media without sea salt, against *S. aureus* and *K. rhizophila as well and E. faecalis*, *E. coli* and *P. aeruginosa*. Moreover, *Z. pseudomaritima* showed positive results in both conditions, but interestingly only against all Gram-positive bacteria tested. Other example of antibacterial activity in both conditions is *L. purpurea* against *K. pneumoniae*. However, *L. maritima* did not present positive results against Gram-positive and negative bacteria tested in this work.

4. Discussion

4.1 Enzymatic screening

a) Cellulolytic activity

Extracellular enzymes with cellulolytic activity were produced in all isolates, especially in the non-salty media (where there were only positive records). These results can be justified by the fact that the fungi under this study are lignicolous. The external secretion of degrading enzymes plays a crucial role in the deterioration of organic matter, as well as in colonization and fixation to the host (Hankin and Anagnostakis, 1975). Marine fungi specialized in colonizing wood possibly use the activity of cellulases to degrade the cell wall of the plant to allow its attachment to the wooden substrate (Hyde et al. 1998). The recording of cellulolytic enzyme activities can be an indicator that the type of decay in these substrates is soft rot, characterized by the degradation of cellulose, hemicellulose and lignin (Eaton and Hale, 1993). Bearing in mind that they are part of a group of microorganisms known to be one of the major wood decomposers in the marine ecosystem (Kohlmeyer et al. 1995), it makes sense that the tested isolates show this type of enzymatic activity.

However, only half of the isolates showed this activity in salty media. Byrne and Eaton (1972) and Kohlmeyer and Kohlmeyer (2013) proved that the decomposition of wood by marine fungi is slower in media with higher salinity compositions. Venâncio et al. (2017)

proved the same, however using terrestrial fungal species. Remembering the importance of marine fungi in the decomposition of lignocellulose in the marine ecosystem, the degradation process is probably delayed by the presence of NaCl. In the experiment carried out, cellulolytic enzymatic production would possibly be observed in media with 3% sea salt, if the incubation time in these media was longer.

b) Pectinolytic activity

Enzymes with this activity are able to break down pectin molecules, structural components of plant cell walls (Poveda et al. 2018). Widely used in the food industry, practically all commercial pectinolytic preparations are produced by fungi (Singh et al. 1999), with emphasis on *Aspergillus niger* (Gummandi and Panda, 2003). Balabanova et al. (2018) also indicate that marine fungi could be used for bioremediation, due to the potential use of polymeric substrates as sources of carbon, relying on pectinolytic enzymes.

With the exception of the isolates *Sedecimiella taiwanensis* and *Lulworthiopsis maritima* in non-salty medium, pectinase activity was recorded in all tests, with salinity not being an inhibitory factor. Pectinase-type enzymes are already known metabolites produced by marine fungi (Cuomo et al. 1987; Niture et al. 2008; Balabanova et al. 2018; Poveda et al. 2018). Similar to the results related to cellulase activities, pectinases are needed for colonization and breakdown of the substrate in the aqueous environment, since pectin is one of the components present in the host´s middle lamella of the cell wall (Balabanova et al. 2018; Poveda et al. 2018) and pectinases are the first enzymes secreted by fungi when attaching to the plant/wood and could be associated with pathogenesis, when it colonizes a living host (Niture et al. 2008).

The fact that the presence of marine salts did not influence extracellular production may indicate that these marine fungi are euryhalines, being adapted to different levels of environmental salinity that they can be active elements in the degradation of compounds with pectin in the estuarine system (Velho and DeSouza, 1982). Like *Fusarium moniliforme* (Niture et al. 2008), our isolates were able to produce pectinolytic compounds in media without salt.

Sedecimiella taiwanensis did not reveal pectinolytic activity. There is a lack of information regarding the enzymatic activities of this fungus, so this study presents the first results regarding its extracellular activity.

Regarding pectin lyases, the same isolates that produced these enzymes also produced pectinases, under the same salinity conditions. *Halosphaeria submersa* did not produce pectin lyase in any media, despite having produced pectinase, as well as *Lulworthiopsis purpurea* with sea salt. Apart from these exceptions, the production of pectin lyase was accompanied by the production of pectinase.

c) Amylolytic activity

This group of enzymes is able to catalyze the hydrolysis of sugars such as glucose, maltose and fructose, from starch molecules, produced by *Aspergillus* and *Penicillium* species and with applications in various industries, such as detergent production, fuel alcohol, textiles, paper and bakery (Sundarram and Murthy, 2014; Liu and Kokare, 2017). Previous studies have shown that marine fungi are capable of synthesizing enzymes with amylolytic properties (Mohapatra et al. 1998; Burtseva et al. 2003; Sarkar et al. 2010; Homaei et al. 2016).

Only *S. taiwanensis* and *Zalerion maritima* were positive for this activity. Due to the potential use of this enzyme in extracellular digestive processes, these isolates may use enzymes with amylolytic activity as a way of absorbing nutrients, namely cleaved sugars. However, *S. taiwanensis* tested negative in the presence of salinity, which may indicate that this isolate resort to other metabolic pathways in its nutrition, in the marine environment. As mentioned above, there is no information available regarding enzymatic activity of this species or genus. Regarding the genus *Zalerion*, it is known that there are species of this genus that produce amylase (Cuomo et al. 1987).

d) Xylanolytic activity

Xylanases are basically glycoside hydrolase enzymes capable of catalyzing the hydrolyzation of xylan (the second most abundant polysaccharide in plant cells) to xylose (Liu and Kokare, 2017). The most eminent examples of fungal species producing xylanolytic activity are part of the genera *Trichoderma*, *Penicillium* and *Aspergillus*, being the main commercial source of this type of enzymes (Bajpai, 1997; Liu and Kokare, 2017). They are a group of enzymes with varied biotechnological applications: food supplement, bread and various drinks, textiles, bleaching processes and production of other industrial reagents (Bajpai, 2014). Raghukumar et al. (2004), showed for the first time a group of marine fungi

as a potential biotechnological source of xylanase with applications in bleaching of paper pulp.

In our set of tests, only *Zalerion* species tested positive for this activity. These results are in line with some previous studies, where the same activity was observed for *Zalerion* species (Raghukumar et al. 1994; Bucher et al. 2004).

e) Proteolytic activity

The protease group consists of enzymes that catalyze hydrolysis in covalent peptide bonds (Liu and Kokare, 2017) with an extensive industrial utility, from the food industry to the textile, pharmaceutical, bioremediation and biobleaching of proteins (Souza et al. 2015). Two of the enzymes tested in this work are part of this group: caseinase and gelatinase.

Halosphaeria submersa and *S. taiwanensis* produced caseinolytic activity in both saline conditions. *Lulworthiopsis maritima* and *Z. pseudomaritima* only produced with and without salinity, respectively. Caseinase was previously identified as an egg mass degrading enzyme in some species of fish (Lee et al. 2005). *Aspergillus*, *Penicillium* and *Cladosporium* sp. with this activity have been isolated from fish eggs (Park et al. 2018).

In the screening of gelatinolytic activity, the same isolates that presented caseinolytic activity independent of salinity also showed positivity for gelatinase, however only in the absence of salt. Pisano et al. (1964) had already shown the capabilities of a *Halosphaeria* sp. produce gelatinase in non-salty conditions, as observed in this work. Due to the correlation that these two activities had in these specific isolates, there is a possibility that these metabolic pathways are, in some way, associated. To determine this, further efforts will be needed to prove it.

f) Ureolytic activity

Urea represents one of the sources of nitrogen that supports growth in marine fungi (Sguros et al. 1973). Metalloenzymes like urease, catalyze the hydrolysis of urea to carbon dioxide and ammonia (Kumari et al. 2016). Ureolytic microorganisms like marine fungi are capable of precipitate carbonates and could be used for biorecovery of valuable metals disseminated in the environment (Li and Gadd, 2017).

Only *H. submersa*, *S. taiwanensis* and *Z. maritima* (medium without salinity) tested positive for this activity. Information on ureolytic activity in these genera is scarce, and these results are the first evidence of this activity in these fungi.

Considering that nitrogen is an essential nutrient for fungal growth, two hypotheses are presented to justify the lack of ureolytic activity in the other isolates: either they can acquire this nutrient through another metabolic pathway that can reduce other molecules in nitrogen; or the experimental conditions performed were not optimized for these specific genera.

g) Chitinolytic activity

Chitin is a chain molecule formed by N-acetylglucosamine polymers and a structural component of fungal cell walls, as well as in exoskeletons of several marine invertebrates and can be degraded by chitinase into monomers (Kumar, 2000; Muzzarelli, 2013; Liu and Kokare, 2017). This type of enzymes can be used as antifungal agents and biopesticides, they are also useful for the manufacture of pharmaceutical products, they can be used as tools in cell engineering and in medical diagnosis (Dahiya et al. 2006). Some marine fungi capable of synthesizing chitinase are already known, such as *Aspergillus*, *Rhizopus*, *Penicillium* and *Beaueria* (Raghukumar, 2017b).

Only *H. sumersa* produced this enzyme, in both conditions. This isolate is capable of producing cellulases, pectinases and chitinases, which makes it a fungus to be explored for a potential application in synthesis of antifungal compounds, as it presents a spectrum of enzymatic activities applicable to this biotechnological area.

h) Laccase activity

Laccase is a ligninolytic enzyme, important in lignin degradative processes with applications in bleaching in the wood, paper and pulp industries (Raghukumar et al. 1994). Several marine fungi have been reported to produce laccase activity, like *Penicillium sp., Z. varium*, *A. sclerotiorum*, *Cladosporium cladosporioides* and *Mucor racemosus* (Raghukumar et al. 1994; D'Souza et al. 2006; Bonugli-Santos et al. 2010; Raghukumar et al. 2008).

None of the tested isolates showed positive results for laccase activity. According to the results in Raghukumar et al. 1994, there are several experimental parameters that can restrict the production of laccase in marine fungi. Two of the most evident laboratory conditions are the pH levels and the nitrogen concentration of the culture medium. For the same conditions,

there were different results, which varied according to the species under test. The fact that there was no such activity, may indicate that the conditions of the culture media were not optimized for the species used in the screening. To find out if these isolates have the capacity to produce laccase, other media with different conditions should be tested.

4.2 Antibacterial activity screening

Much of the existing literature on antibacterial activities in the Kingdom Fungi comes from terrestrial fungi. However, there has been an increased interest in research on antibacterial compounds in fungi marine species in recent decades (Belofsky et al. 1998; Samuel et al. 2011; Devi et al. 2012; Deshmukh et al. 2018). Considering that there is still a large gap in the knowledge of antibacterial properties of marine fungi, further studies may bring clarification and discoveries within this subject (LiBerra and Lindequist, 1995; Samuel et al. 2011).

Regarding Gram-positive bacteria, *Staphylococcus aureus*, *Kocuria rhizophila* and *Enterococcus faecalis* are often pathogenic to humans, all being an emerging danger and causing nosocomial infections when the host's immune system declines and being reported for developing resistance to many antibiotics used in medical therapies and treatments (Lowy, 1998; Le Loir et al. 2003; Sood et al. 2008; Indarmawan et al. 2016; Kandi et al. 2016).

Zalerion pseudomaritima proved to have an extremely effective activity against Gram-positive bacteria, both in the presence and absence of sea salts, by inhibiting all Gram-positive strains tested in this work. Furthermore, *Zalerion maritima* (with salinity), *L. purpurea* (without salinity) and *M. halima* (both conditions) also showed antibacterial activity against *S. aureus*. In Noble et al. (1991), the antibacterial capacity of a compound produced by *Z. arboricola* was tested on *S. aureus* strains, however, no positive results were obtained. Moreover, there is no further information on any type of antibacterial screening for *S. aureus* from *Zalerion* species. The same is not true of *Papulaspora* species, the genus to which *M. halima* previously belonged to. *Papulaspora pallidula* and *P. immersa* are two species described to be able to inhibit *S. aureus* cells (Muhsin and Hachim, 2016). *Zalerion pseudomaritima*, *Z. maritima* and *M. halima* inhibited the growth of *K. rhizophila* in our study. Having been independent of salinity conditions, this is the first record of antimicrobial activity of *Zalerion* sp. against *K. rhizophila*. In antibacterial activity against *E. faecalis*, *Z.*

maritima and *M. halima* also tested positive (without salt in both cases). These results are also the first records of this activity in these marine fungi species.

The screening of the isolate *Z. pseudomaritima* presented curious results. It inhibited only Gram-positive bacteria and 100% of those tests were positive: it is excellent against Gram-positive and useless against Gram-negative. One possible explanation is due to the different composition of Gram-positive and negative bacterial cell walls. It is known that there are antibiotics more suitable for Gram-negative and others to Gram-positive bacteria. In Indarmawan et al. (2016), extracellular proteases produced by a marine fungus resulted in an antibacterial activity against only Gram-positive bacteria. The solution to the antibiotic resistance of these bacteria may be in the production of this type of extracellular fungal proteases or other peptides that bind and inactivate the cell wall biosynthesis. *Zalerion pseudomaritima* and *M. halima* could be a new unknown source of these compounds and a possible tool in the fight against multi-resistant Gram-positive bacteria. A more focused study of these fungi may give rise to the discovery of one or more promising compounds to fight infections with Gram-positive bacteria, regardless of salinity.

Most strains of *E. coli* present in the normal microbiota are harmless and prevent the colonization of other pathogenic bacteria to their host (Nataro and Kaper, 1998). However, *E. coli* serotypes can cause serious infections, such as food poisoning, meningitis, gastroenteritis, urinary infections and Crohn's disease (Costin et al. 1964; Nataro and Kaper, 1998; Kaper et al. 2004). *Klebsiella pneumoniae* and *P. aeruginosa* are emerging opportunistic pathogenic microorganisms in nosocomial diseases with large fatality rates, both species being reported for their multi-resistance to a variety of antibiotics (Van Delden and Iglewski, 1998; Borer et al. 2009; Wong et al. 2012; Gellatly and Hancock, 2013; Martin et al. 2018) representing a real threat to global health systems. Interestingly, only *M. halima* revealed activity against *E. coli* in this work. *Lulworthiopsis purpurea* effectively inhibited the growth of *K. pneumoniae* in both conditions, thus being the first evidence of this activity from this genus (simultaneously encompassing *Lulworthia* sp.). Also, *Sedecimiella taiwanensis* tested positive in the absence of salt. Regarding the *P. aeruginosa*, it was the bacteria that was least affected by the marine fungal isolates. Only *M. halima* in the absence of salinity was able to inhibit this multi-resistant strain.

In Kong et al. (2017) three new compounds (penicitor A, aculene E and penicitor B) were isolated from fermentative processes of a *Penicillium* sp. of marine origin, proving to be

promising due to significantly reducing the activity of virulent Gram-negative bacteria. In the same way, *L. purpurea*, *M. halima* and *S. taiwanensis* can inhibit pathogenic Gram-negative bacteria as mentioned above and potentially could produce effective antibiotics against this type of bacteria. Finding fungal isolates capable of inhibiting extremely virulent and pathogenic bacterial strains (like *K. pneumoniae* and *P. aeruginosa*) represents an alternative to combating them.

Since the discovery of penicillin, many other compounds of fungal origin have been applied to many sectors of human interest, one of which is the medicinal sector, where there is a strong application of antibacterial compounds from fungi, including marine species (Devi et al. 2012). Some of these compounds have been shown to be inhibitory or even eradicating many multi-resistant bacterial strains with a devastating clinical record in human patients, over many years and globally (Xu et al. 2015).

In this work with marine lignicolous fungi, we demonstrated some antibacterial activity in multi-resistant and pathogenic bacterial isolates. In future, it is necessary to study more fungi marine species and specially lignicolous species with regard to the production of antimicrobials that could give us new effective compounds in the fight against nosocomial infections and other bacterial diseases that affect global health.

To finalize, some factors may have influenced antibacterial inactivity, such as the shortened incubation time as theorized in Hagestad et al. (2019). In this study, fungi were incubated with bacteria for only 24 hours, which may "possibly not allowing all fungi to properly respond to the presence of the bacteria". The incubation temperature was also discussed in Hagestad et al. (2019) as another factor against the synthesis of compounds. Huang et al. (2011), showed that the variation in salinity concentration is capable of affecting the final number of metabolites produced. The optimal range of salinity for growth is between 3 and 6% NaCl (Huang et al. 2011), enabling the hypothesis that the concentration used in the cultures of this study may not have been the most profitable. Possibly with an intermediate concentration, the results may be more expressive.

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Chapter IV

Final considerations and Future perspectives

Final considerations and future perspectives

Originally, the idea for this work was born out of pure curiosity. The foundation of this study is based on one of the most primitive forms of science: questioning. As observant beings and with saline channels running through the city, first came the core question, which was "What will be found if some blocks of wood were submerged in the Ria de Aveiro?". From there, the next step was followed by the elaboration of the experience. The opportunity arose to sink ten blocks of wood in a marina, in the estuary of Ria de Aveiro, courtesy of a colleague from the Department of Biology, Claúdio Brandão, who made his personal space available to facilitate our experience. The idea emerged and this work saw the daylight. Hundreds of hours in the lab and the results came. In those samples, seventeen different species of marine fungi capable of colonizing wood were identified, three of which turned out to be proposals for novel species. After exhaustive phylogenetic studies, two genera have been reevaluated to improve the taxonomic organization of these ecological group. All of this was possible in just one sampling location. This is the great weakness of this study. It was not possible to compare communities from different locations, with different environmental conditions, such as salinity gradients, immersion time, light penetration, currents and agitation of the water mass, proximity to land environments, among others. From these doubts, a new question arises: "What will be found if some blocks of wood were submerged in the Ria de Aveiro, spread over several strategic locations?". In other words, the weak point may give rise to other possible future works on lignicolous marine fungi: Ria de Aveiro is a complex ecosystem with many mysteries, yet to be solved. This provided an opportunity for further studies, outside of diversity, such as biotechnological potential. This is one of the strengths of this work, as it has added relevant information for the study of these microorganisms. Possibly, if more sites had been sampled, there would have been no laboratory time to perform this part. What was more negative about the work, it became a positive aspect, all due to curiosity and determination to experiment.

Concluding this dissertation, just as it started: the aquatic microscopic world is a vast unknown universe; as we discover, we realize that we know less and less... The solutions to many problems that affect our present day may be hidden under our waters and only the scientist who is able to question and experiment is the one who is able to unveil them.