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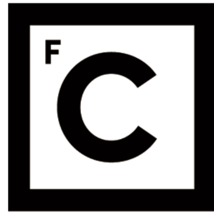

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ULisboa

Marine fungal community associated with standing plants of *Spartina maritima* (Curtis) Fernald

Doutoramento em Biologia

Especialidade em Microbiologia

Maria da Luz Jeremias Cardinha do Maio Calado

Tese orientada por:

Prof. Doutora Margarida Barata e Prof. Doutor Ka-Lai Pang

Documento especialmente elaborado para a obtenção do grau de doutor

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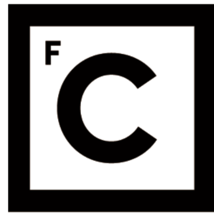

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RESUMO

A presente tese pretendeu contribuir para o aumento do conhecimento relativo às comunidades de fungos marinhos superiores que colonizam ecossistemas intertidais temperados, dada a escassez de estudos desenvolvidos neste domínio em geral, e em Portugal em particular. Especificamente pretendeu-se inventariar as espécies fúngicas associadas a uma das macrófitas mais importantes e dominantes dos sapais da costa portuguesa, a *Spartina maritima* (Curtis) Fernald, e compreender melhor a dinâmica da comunidade e diversos aspectos da ecologia de cada espécie fúngica.

O estudo foi desenvolvido em dois sapais (Castro Marim e Ria de Aveiro) com características distintas, no que respeita à localização geográfica, configuração física, estado de conservação e representatividade da planta hospedeira.

O sapal de Castro Marim situa-se no troço final do estuário do Guadiana, na costa sudeste de Portugal (37.23° N, 7.42° W), e está incluído numa Reserva Natural; apresenta, por isso, estatutos especiais de protecção e uma comunidade de *S. maritima* bem conservada, que se distribui paralelamente ao rio numa faixa contínua. O sapal da lagoa costeira da Ria de Aveiro localiza-se na costa noroeste de Portugal (40.62° N, 8.74° W) e integra uma complexa rede de canais sujeita a fortes pressões antrópica; nesta área, a comunidade de *S. maritima* está muito fragmentada e dispersa ao longo da faixa de vegetação.

A amostragem da comunidade de fungos marinhos envolveu a recolha de 195 plantas inteiras, maduras, enraizadas e em posição natural de *S. maritima* durante 2 anos (de Outubro de 2010 a Agosto de 2012), com uma periodicidade de 2 meses; recolheram-se 20 plantas de cada área de estudo nos primeiros 3 meses, e 15 plantas nos restantes períodos. Em laboratório, cada planta, previamente lavada e seca ao ar, foi separada em nove categorias de substrato, de acordo com as estruturas vegetais que a compunham e estado fisiológico das mesmas; especificamente, em bainhas vivas, bainhas senescentes, bainhas em decomposição, caules vivos, caules senescentes, caules em decomposição, limbos vivos, limbos senescentes e limbos em decomposição. As denominações "vivo", "senescente" e "em decomposição", caracterizaram tecidos verdes, amarelos e acastanhados, com estrutura física nada, pouco ou bastante alterada respectivamente.

A identificação dos fungos marinhos recorreu a dois métodos distintos, mas complementares; (1) a observação directa de estruturas fúngicas (esporos, estruturas de frutificação e hipopódios) e (2) a sequenciação da região ITS (Internal Transcribed Spacers) do DNA ribossomal nuclear (rDNA).

O primeiro método envolveu a análise individual de cada substrato vegetal à lupa; as estruturas fúngicas detectadas nesse substrato foram observadas ao microscópio e identificadas com base nas suas características morfológicas, recorrendo a chaves dicotómicas específicas para identificação de fungos marinhos. Adicionalmente registou-se a distribuição vertical e densidade das estruturas fúngicas de cada espécie. No período de Fevereiro de 2012 a Agosto

de 2012, foram ainda recolhidas 5 plantas extras, as quais foram lavadas e imediatamente observadas. Algumas estruturas de frutificação (ascocarpos ou picnídios) diferenciadas neste material vegetal fresco foram extraídas e utilizadas na obtenção de culturas puras, através do método de esporo único. Os fungos isolados foram preservados por três métodos distintos: (1) crescimento activo em Corn Meal Agar (CMA) preparado com água do mar diluída (50%), a 4 °C; (2) discos de micélio imersos em água do mar estéril (50%), a 4 °C; e (3) discos de micélio imersos em solução aquosa de glicerol (10%), a -80 ºC. Duas culturas puras de cada espécie, de cada local, foram seleccionadas para determinar a taxa de crescimento vegetativo em CMA preparado com água destilada e água do mar diluída (50%).

Após esta análise, agrupou-se o material vegetal pertencente à mesma categoria de substrato e proveniente de todas plantas recolhidas no mesmo período de amostragem e local de estudo. Todos os substratos vegetais foram posteriormente liofilizados.

A identificação molecular dos fungos marinhos foi realizada apenas a partir dos substratos recolhidos no primeiro ano de amostragem. A metodologia molecular envolveu, numa primeira fase, a extracção do DNA nuclear das culturas puras, e amplificação e sequenciação da região ITS do rDNA; estas sequências constituíram uma colecção de referência para comparação e identificação dos fungos presentes nas amostras vegetais. Posteriormente, o DNA nuclear dos 88 substratos vegetais foi igualmente extraído, e a mesma região genómica amplificada por primers com especificidade para fungos. Os amplicões obtidos de cada substrato foram clonados no sentido de isolar cada sequência ITS; no total, obtiveram-se 1037 clones. No sentido de seleccionar os clones de DNA recombinante representativos de cada biblioteca da região ITS de cada substrato vegetal, foi realizada uma análise de perfis de restrição (RFLP); os diferentes perfis de restrição de cada biblioteca foram sequenciados.

Estas sequências foram comparadas com sequências depositadas na base de dados pública internacional GenBank e com as sequências da colecção de referência (culturas puras), e identificadas até à menor categoria taxonómica possível. A identificação destas sequências permitiu a extrapolação e identificação das restantes sequências extraídas de todos os substratos vegetais analisados.

A conjugação dos métodos morfológico e molecular revelou-se fundamental para o conhecimento da diversidade dos fungos marinhos superiores associados a *S. maritima*, na medida em que permitiu confirmar ou corrigir as identificações dos fungos mais comuns realizadas por cada um dos métodos e complementar o inventário com as espécies mais infrequentes. De uma maneira geral, houve uma concordância entre os dois métodos no que respeita à representatividade de cada fungo nas comunidades, nos substratos vegetais e nas áreas de estudo. No total, foram identificados 45 fungos nas primeiras fases do processo de decomposição de plantas de *S. maritima*. Tal como em estudos semelhantes anteriores desenvolvidos em sistemas intertidais, esta comunidade revelou ser dominada por fungos pertencentes ao filo Ascomycota e à classe Dothideomycetes.

A comparação das comunidades de fungos marinhos associadas a *S. maritima* e outras espécies de *Spartina* evidenciou a existência de um grupo nuclear (core group) de espécies

que surge associado a este genéro de plantas hospedeiras, independentemente da sua localização geográfica. Este grupo inclui espécies em associação exclusiva com plantas do género *Spartina*, como *Anthostomella spissitecta*, *Byssothecium obiones*, *Buergenerula spartinae*, *Mycosphaerella* sp. I, *Phaeosphaeria halima*, *Phaeosphaeria spartinicola*; espécies que colonizam igualmente outras plantas intertidais de climas temperados, como *Leptosphaeria marina* e *Sphaerulina orae-maris*; e espécies cosmopolitas, que surgem em diversos substratos de climas tropicais e temperados, como *Aniptodera chesapeakensis* e *Dictyosporium pelagicum*. Apesar dos fungos *Lulworthia* sp. 1 e *Stagonospora* sp. 1 não terem sido identificados até à espécie, estes géneros taxonómicos são comumente observados em plantas de *Spartina*.

As espécies *B. obiones*, *B. spartinae*, *Mycosphaerella* sp. I, *P. halima* e *P. spartinicola* foram registadas como muito frequentes na comunidade geral amostrada neste estudo e presentes em todos ou na maioria dos períodos de amostragem, em concordância com estudos semelhantes realizados com outras espécies de *Spartina.* Este estudo revelou que existem, no entanto, espécies muito frequentes nas comunidades associadas a *S. maritima* mas ausentes noutras espécies de *Spartina*, como o fungo cosmopolita *Natantispora retorquens*, e vice-versa.

As espécies infrequentes *Anthostomella spissitecta, Camarosporium roumeguerii*, *Ceriporia lacerata*, *Coniothyrium obiones*, *Cryptococcus mangaliensis, Decorospora gaudefroyi*, *Erythrobasidium hasegawianum*, *Halosarpheia trullifera*, *Leptosphaeria marina*, *Penicillium chrysogenum e Stagonospora haliclysta* foram registadas pela primeira vez em associação com o género *Spartina* e/ou com *S.maritima*.

A presença ou ausência das espécies nas comunidades fúngicas associadas à mesma ou outra espécie de *Spartina* poderá estar relacionada com diferenças na estrutura física e composição química das plantas hospedeiras e/ou factores macro ou microambientais.

Os fungos marinhos nas duas comunidades amostradas neste estudo e particularmente os mais frequentes apresentaram padrões de distribuição verticais específicos nas plantas de *S. maritima* em posição natural, i.e. localizavam-se na mesma posição vertical relativa. A posição relativa na planta parece reflectir o grau de adaptação das espécies fúngicas às condições marinhas. As diferentes estratégias de reprodução sexuada e assexuada ao longo das plantas representam e evidenciam algumas das estratégias adoptadas pelos fungos marinhos para se adaptarem; os fungos incluídos na classe Sordariomycetes, com ascos unitunicados e mecanismos passivos de libertação de esporos, dominam as partes basais das plantas, enquanto os fungos incluídos na classe Dothideomycetes, com ascos bitunicados e mecanismos activos de libertação de esporos, ocupam principalmente as partes aéreas. Os dados sugerem que os fungos que surgem nas partes basais das plantas são marinhos obrigatórios, enquanto os fungos das partes superiores são marinhos facultativos. As partes intermédias representam, por isso, uma zona de transição e sobreposição dos fungos que colonizam exclusivamente os ecossistemas marinhos e os que podem provir de ecossistemas fluviais ou terrestres. Apesar de revelarem diferentes dependências e tolerâncias à salinidade em condições naturais, todos estes fungos dominantes cresceram em meio de cultura sem

salinidade, mas com uma taxa de crescimento mais elevada em meio de cultura preparado com água do mar. Estes resultados evidenciaram a elevada plasticidade destes fungos de se adaptarem a diferentes condições ambientais.

As espécies dominantes da comunidade foram também as que exibiram uma área de distribuição vertical mais extensa, e que surgiram em mais de uma estrutura vegetal, nos diferentes estados fisiológicos, ao longo de todo o período de amostragem. A presença destas espécies sapróbias em tecidos vegetais vivos sugere que estas possam iniciar o processo de colonização como endófitas.

A conjugação de todos os resultados indicia que os padrões de distribuição vertical, e a ocorrência e o papel ecológico dos fungos mais frequentes dependem da fase do ciclo de vida da planta e disponibilidade dos substratos vegetais, das condições microambientais dos substratos e adaptação aos ciclos de submersão-emersão, e dos potenciais fungos competitores. Durante o processo de decomposição de *S. maritima*, os fungos marinhos obrigatórios *B. obiones*, *Lulworthia* sp. 1 e *N. retorquens* assumem um papel ecológico mais activo na decomposição das bainhas e caules inferiores; o fungo marinho facultativo *Mycosphaerella* sp. I, na decomposição dos limbos superiores; os fungos marinhos facultativos *P. halima* e *Stagonospora* sp. 1, na decomposição das bainhas e limbos superiores; e os fungos marinhos facultativos *P. spartinicola* e *B. spartinae*, na decomposição de todas as estruturas vegetais.

Em suma, este estudo contribuiu para um enriquecimento do conhecimento da composição específica, diversidade e dinâmica das comunidades de fungos marinhos superiores associadas a plantas maduras, enraizadas e em posição natural de *S. maritima*, bem como dos requisitos e papel ecológicos de cada espécie na decomposição destas plantas hospedeiras.

Palavras-chave: fungos marinhos; *Spartina maritima*; decomposição; requisitos ecológicos; potencial papel ecológico

ABSTRACT

The major purpose of this thesis was to complement the current knowledge regarding marine fungal communities and particularly those inhabiting Portuguese temperate salt marshes. Specifically, this study mainly intended to assess the species composition and diversity of the fungal communities associated with one of the most dominant macrophytes in these ecosystems, *Spartina maritima* (Curtis) Fernald, and to contribute to a better understanding of community dynamics and key ecological aspects of the fungi. The study was conducted in two geographically and physically distinct salt marshes, Castro Marim and Ria de Aveiro, where 195 mature, standing live plants were collected over a 2-year period (October 2010 to August 2012) from each study site. Each air-dried plant was separated into nine substrate categories according to the vegetative structure (leaf sheaths, stems and leaf blades) and physiological state of each structure (live, senescent and decaying). Identification of marine fungi was performed by two distinct, but complementary methods, i.e. direct observation of fungal structures (fruit bodies, spores and hyphopodia) and sequencing of the internal transcribed spacer regions of rDNA (ITS). The first method involved an individual observation of each substrate under dissecting- and light microscopes for detection of fungal structures; fungal taxa were morphologically identified using specific dichotomous keys for marine fungi. The vertical position and density of fruiting structures produced by each identified fungus was also recorded. The most frequent fungi were isolated in pure cultures by single spore method. Plant materials from the same substrate category, sampling period and study site were mixed and freeze-dried. Only the plant samples from the first sampling period were used for molecular identification of fungi. This second method involved DNA extraction of pure fungal isolates and plant samples, and amplification of the ITS region. Amplicons from plant samples were cloned in order to isolate individual amplicons of mixed PCR products. ITS sequences of the 1037 clones obtained from the plant samples were submitted to a restriction fragment length polymorphism analysis (RFLP); clones with different digestion profiles were sequenced. Phylogenetic analyses were performed with sequences of clones, fungal isolates and BLAST best-hits. A comparison between morphological and molecular methods revealed a general agreement in taxonomic assignments and representativeness of each fungus in the community, vegetative structure and study site. The combination of both methods was demonstrated to be crucial for a more realistic and accurate representation of the fungal community. Forty-five fungal taxa were recorded in *S. maritima* samples; 91% of these were filamentous ascomycetes, included in the Dothideomycetes and Sordariomycetes. The majority of the fungal species most frequently recorded in this study were previously described from other species of *Spartina*. Nevertheless, the studied fungal community also included other infrequent species that represent new records for the genus *Spartina* and/or *S. maritima* plants, e.g. *Anthostomella spissitecta, Camarosporium roumeguerii*, *Ceriporia lacerata*, *Coniothyrium obiones*, *Cryptococcus mangaliensis, Decorospora gaudefroyi*, *Erythrobasidium hasegawianum*, *Halosarpheia trullifera*,

Leptosphaeria marina, *Penicillium chrysogenum* and *Stagonospora haliclysta*. The presence or absence of species in fungal communities may be related with intra- and interspecific differences in the physical structure and chemical composition of the host plants and/or macro and microenvironmental factors.

Similarly to other grass-like plants, the results also demonstrated that the marine fungi are vertically distributed along standing plants of *S. maritima*. Moreover, the most frequent fungal taxa exhibited wide vertical distribution ranges, a high investment in the production of fruiting structures and were present during all the sampling period on senescent and decaying vegetative structures. The majority of these fungi were also found on live plant tissues, which indicated that these saprobic species might initiate the colonisation of plant substrates as endophytes. These findings suggested that the vertical distribution patterns, and occurrence and ecological role of most frequent fungi depend on the phase of plant life cycle and substrate availability, micro-environmental conditions of substrates and adaptation to submersion/exposure cycles, and potential fungal competitors. During the decay process of *S. maritima,* the obligate marine fungi *Natantispora retorquens*, *Byssothecium obiones* and *Lulworthia* sp.1 seem to be involved in the complete decomposition of lower leaf sheaths and stems; facultative marine fungi *Mycosphaerella* sp. I, of leaf blades; facultative marine fungi *Phaeosphaeria halima* and *Stagonospora* sp. 1, of upper standing leaves; and *Buergenerula spartinae* and *Phaeosphaeria spartinicola*, of all vegetative structures.

Key words: marine fungi; *Spartina maritima*; decomposition; ecological requirements; potential ecological role

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THESIS FRAMEWORK

This thesis is organized into four chapters.

Chapter 1 (General Introduction) comprises an overview of the current knowledge concerning marine fungi in general (Marine fungi – a brief review), and fungi inhabiting salt marshes in particular (Salt marsh fungi). This last review was included in the recently published volume "Marine Fungi and fungal-like organisms", as a book chapter. This introduction also addresses the main gaps and limitations to the understanding of marine fungal communities and the objectives of the present study.

The original research work performed in this study was included in two papers published in international scientific journals, which represent chapters 2 and 3. The structure and original content of the published papers were maintained in the essential but the formatting style was changed; all the chapters follow the same formatting and bibliographical rules.

Chapter 2 (**Diversity and ecological characterization of sporulating higher filamentous marine fungi associated with *Spartina maritima* (Curtis) Fernald in two Portuguese salt marshes**) presents an inventory of fungal taxa associated with standing plants of *Spartina maritima* assessed by morphological identification of fungal structures. The biotic and abiotic factors that may determine the presence and abundance of fungal species on *Spartina maritima* and their distribution along the vertical axis of these host plants are also enumerated.

Chapter 3 (**Ecological preferences of marine fungi associated with standing decaying plants of *Spartina maritima* (Curtis) Fernald**) presents the results from the molecular identification of fungi on live, senescent and decaying leaf sheaths, stems and leaf blades of standing plants of *Spartina maritima*. In this chapter, the molecular and morphological methods adopted in this study are compared. The presence and prevalence of fungi on each plant substrate are used to assess their ecological preferences and infer about their ecological role in the decay of *Spartina maritima*.

Chapter 4 (Final overview) highlights the main results of this study, pointing out some knowledge gaps that should be approach in future studies.

Over the past 6 years, many new species have been described from marine habitats and many taxonomic ambiguities have been resolved as a result of the application of more accurate and improved molecular tools. This has led to dramatic changes in nomenclatural rules and taxonomic classification of fungi after the chapter "Salt marsh fungi" and the paper "Diversity and ecological characterization of sporulating higher filamentous marine fungi associated with *Spartina maritima* (Curtis) Fernald in two Portuguese salt marshes" were written. The terms anamorph and teleomorph were replaced to asexual and sexual morphs respectively, and sequenced asexual morphs were transferred from the artificial group "anamorphic fungi" to different taxonomic categories within the Ascomycota and Basidiomycota; holomorphic fungal

species, for which both sexual and asexual morphs were demonstrated to be connected, were designated by only one name. These new rules were followed in the paper "Ecological preferences of marine fungi associated with standing decaying plants of *Spartina maritima* (Curtis) Fernald".

CHAPTER 1 - General Introduction

1.1 Marine fungi – A brief review

Marine fungi represent an ecological group of fungi that occur from inshore regions to deep oceanic waters (Fell and Newell 1998; Hyde et al. 1998), composed primarily by higher filamentous fungi included in the Basidiomycota (Ustilaginomycetes and Agaricomycetes) and Ascomycota (Dothideomycetes, Eurotiomycetes, Laboulbeniomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes, Arthoniomycetes and Sordariomycetes) (Kohlmeyer and Kohlmeyer 1979; Hyde et al. 2000; Jones and Pang 2012). Most marine fungi belong to the Dothideomycetes and Sordariomycetes, particularly to Halosphaeriaceae (Jones et al. 2009; Sakayaroj et al. 2011; Jones and Pang 2012; Pang 2012).

Marine fungi include species with a wide range of nutritional modes, i.e. fungal species that establish a parasitic or symbiotic mycorrhizal, lichenoid or endophytic relationship with several hosts, and saprobes on dead organic material of plant and animal origin (Kohlmeyer and Kohlmeyer 1979; Hyde et al. 1998; Kohlmeyer et al. 2004; Jones 2011a; Richards et al. 2012). The majority of marine fungal species are decomposers of plant materials, particularly of woody substrates (Kohlmeyer and Kohlmeyer 1979; Hyde et al. 1998; Jones 2000; Pointing and Hyde 2000). Inherent to their metabolic activities, saprobic marine fungi, especially filamentous ones, play an important functional and ecological role in the nutrient recycling and energy flow in marine ecosystems (Newell 1993, 1996; Hyde and Lee 1995; Hyde et al. 1998; Newell and Porter 2000; Pang and Jones 2012). The strategy adopted by mycelial fungi implicates a penetrating growth mode by expanding hyphal tips combined with enzyme´s activity (Torzilli and Andrykovitch 1986; Newell 1996; Lyons et al. 2003; Raghukumar 2004b). Marine fungi are widely recognized by the diverse range of extracellular biologically-important enzymes involved in the degradation of recalcitrant cell wall materials, such as cellulases, laccases, lignin peroxidases and Mn-dependent peroxidases (Gessner 1980; Torzilli and Andrykovitch 1986; Bergbauer and Newell 1992; Newell et al. 1996b; Pointing et al. 1998; Raghukumar 2002, 2004a; Lyons et al. 2003; Raghukumar 2004b; Jones 2011a). Biotechnological potential of lignocellulolytic marine fungi in bioremediation has been widely investigated (Newell et al. 1996b; Raghukumar 2002, 2004a; Raghukumar 2004b; Jones 2011a). In addition, marine fungi represent an important source of structurally unique bioactive secondary metabolites with antimicrobial, anticancer, anti-inflammatory and analgesic properties (Bugni and Ireland 2004; dela Cruz et al. 2006; Schulz et al. 2008; Jones 2011a; Ebel 2012; Singh et al. 2012; Overy et al. 2014a). The production of these metabolites is species-specific (dela Cruz et al. 2006) and apparently is not affected by the geographical origin of species (Schulz et al. 2008). Eighty out of over 1000 metabolites that have been characterized to date were isolated from marine fungi (Overy et al. 2014a). Although the reasons associated with the production of these metabolites are not totally known, one of the main purposes might be a chemical defense strategy in response to interference competition (Pointing et al. 2000; Jensen and Fennical 2002;

Panebianco et al. 2002); some of these compounds were demonstrated to limit spore germination or fungal growth (Miller 2000). For endophytes, these metabolites might also play an important role in the communication with host species and for adaptation of the hosts to environmental stress (Meng et al. 2011).

Nevertheless their trophic strategy, marine fungal species are physiologically and morphologically adapted to marine environments.
Given the high salinity level of marine environments, marine fungi exhibit different mechanisms in order to maintain homeostasis in their cells; some fungal species synthesize or absorb compatible solutes from surrounding water to their cytoplasm and accumulate them in compartmentalized vacuoles, while others pump sodium ions out of cells (Jennings and Garrill 2000; Jones 2000). Most marine fungi show an optimal vegetative growth and synthesis of secondary metabolites in a saline medium than without any marine salts (Masuma et al. 2001; dela Cruz et al. 2006; Huang et al. 2011; Pang et al. 2011; Overy et al. 2014a).
Marine filamentous fungal species, particularly ascomycetes, have evolved to adapt to a marine life style; most of these species produce microscopic and enclosed fruiting structures in response to the abrasion caused by waves and shifting sand (Fig. 1), exhibit a passive release mechanism of spores to enhance the dispersal of these structures in an aquatic environment, and differentiate spores with different complexities and shapes of appendages or sheaths to facilitate floating, entrapment and attachment to the substrate (Fig. 1) (Kohlmeyer and Kohlmeyer 1979; Jones 2000, 2011a; Au and Vrijmoed 2002; Pang 2002; Campbell et al. 2003; Sakayaroj et al. 2011; Overy et al. 2014a).

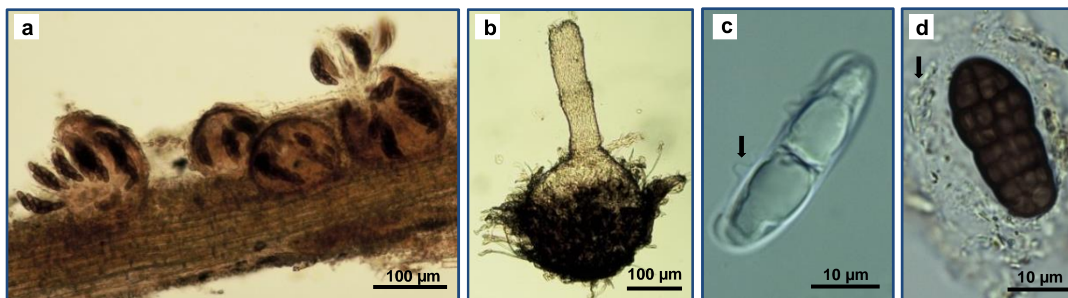


Fig. 1 Fruiting structures of ascomycetous fungi: a) ascomata of *Phaeosphaeria spartinicola*; b) ascoma of *Natantispora retorquens*; c) ascospore of *Natantispora retorquens*; d) ascospore of *Decorospora gaudefroyi* (arrows indicate appendages (c) and sheaths (d))

Ascomycetes with an active discharge of spores and mucilaginous sheaths thrive in intertidal ecosystems (Jones 2000, 2011a; Suetrong et al. 2009; Sakayaroj et al. 2011; Overy et al. 2014a), which are characterized by alternate conditions of water immersion and air exposure associated with daily tidal cycles; these fungi have been demonstrated to have high affinities with terrestrial fungal species (Kohlmeyer and Kohlmeyer 1979; Jones et al. 2009; Schoch et al. 2009a).

Most filamentous ascomycetes possess a delimiting membrane, which prevents the premature expansion of the appendages and sheaths before they are released into the surrounding water (Jones 2011a).

Although the biogeography of marine fungi is still not fully understood (Jones and Pang 2012), some fungal species have been exclusively found in tropical, subtropical or temperate climate regions, while others were found to be cosmopolitan species (Kohlmeyer and Kohlmeyer 1979; Hyde and Lee 1995; Hyde et al. 1998; Jones et al. 1998; Sarma and Hyde 2001; Alias et al. 2010; Jones and Pang 2012).
Among the several factors that could determine the macro-geographical distribution of marine fungi, the availability of substrates for colonization and water temperature and salinity are apparently the most important controlling key-factors (Hyde and Lee 1995; Jones 2000, 2011a; Sarma and Hyde 2001; Kohlmeyer et al. 2004; Jones and Pang 2012).
In a general perspective, fungal diversity increases from polar to tropical climate zones (Hyde and Lee 1995; Jones 2011a, b; Pang et al. 2011), and decreases from intertidal towards offshore environments (Kohlmeyer et al. 2004; Nagahama 2006; Burgaud et al. 2013). Marine fungi colonize preferentially estuarine ecosystems, such as mangroves, salt marshes or other coastline habitats (Fig. 2), where the availability of substrates is higher (Jones 2000, 2011a; Morrison-Gardiner 2002; Nagahama 2006; Alias et al. 2010; Azevedo et al. 2012; Overy et al. 2014b; Rämä et al. 2014) and the physical conditions are more favourable (Burgaud et al. 2013).

Fig. 2 Intertidal ecosystems: a) salt marsh; b) mangrove; c) sandy beach

In contrast, extreme abiotic conditions in deep-sea environments, i.e. high hydrostatic pressure, high salinity and low temperature, oxygen and nutrient concentrations, have restricted the colonisation process to a less number of fungal species (Jones 2000; Dupont et al. 2009; Huang et al. 2011; Singh et al. 2012; Burgaud et al. 2013). Hydrostatic pressure and full-strength seawater are the major limiting factors for growth and metabolic activity of filamentous fungi (Pointing et al. 1998, 1999; Burgaud et al. 2009; Dupont et al. 2009).
Although some of the studies revealed that these ecosystems are mainly inhabited by marine yeasts and fungal-like organisms (Nagahama 2006; Bass et al. 2007; Edgcomb et al. 2011), Damare et al. (2006), Nagano et al. (2010), Singh et al. (2011), Xu et al. (2014) identified also fungal signatures of several filamentous ascomycetes in deep-sea sediments.

At a more reduced scale, the influence of biotic and physical factors in the colonization process of each fungal species, such as tolerance to air exposure or submergence conditions, substrate exclusivity and competitive abilities, varies from species to species (Gessner 1977; Poon and Hyde 1998b; Alias and Jones 2000a, b; Barata 2002, 2006; Panebianco et al. 2002; Buchan et al. 2003; Lyons et al. 2005; Al-Nasrawi and Hughes 2012). According to Jones (2000, 2011b) the presence of many fungi depends on a consortium of factors interacting together.

Even though ascomycetous and basidiomycetous yeasts have been frequently reported from the same marine environments, either free floating or attached to a substrate (Raghukumar 2004b; Gadanho and Sampaio 2005; Nagahama 2006; Edgcomb et al. 2011; Fell et al. 2011; Fell 2012; Jones and Fell 2012), these fungi have been neglected or excluded from this ecological group (Jones et al. 2009; Jones 2011a). This may be attributable to the difficulties in morphological identification of species and in the understanding of their life traits. Many species of yeasts retrieved from intertidal and deep-sea environments were demonstrated to be physiologically and phylogenetically related to terrestrial fungi (Alker et al. 2001; Nagahama 2006; Edgcomb et al. 2011; Fell 2012; Burgaud et al. 2013; Overy et al. 2014a), which raises some doubts about the origin of fungal propagules. Few yeast species have been found to be autochthonous to marine environments, particularly basidiomycetes in deep-sea sediments (Nagahama 2006). Gadanho and Sampaio (2005), Burgaud et al. (2009) and Edgcomb et al. (2011) demonstrated, though, that some yeasts inhabiting sea-floor and/or hydrothermal vent fauna were metabolically and functionally active.

The boundaries between terrestrial/freshwater and marine fungi are not always clear and the definition of marine fungi is still being discussed among the scientific community (Pang and Mitchell 2005; Jones et al. 2009; Overy et al. 2014a). The definition of marine fungi proposed by Kohlmeyer and Kohlmeyer (1979) was, for more than 30 years, the most widely accepted and consensual one. These authors distinguish obligate and facultative marine fungi based on the ecological dependency of fungal species on marine conditions to germinate and/or grow vegetatively, produce and disperse spores or vegetative propagules and reinitiate their life cycle. Obligate marine fungi include the species that grow and sporulate exclusively in a marine or estuarine habitat and are permanently or intermittently submerged, whereas facultative marine fungi include species from freshwater or terrestrial environments able to grow and possibly also to sporulate in the marine habitats. This dependency inferred from an active growth in marine environment has not been, though, easy to test or prove, considering that among the fungi recovered from coastal to offshore marine ecosystems that grew vegetatively in culture medium, some might be present as dormant propagules in those ecosystems (Raghukumar and Raghukumar 1999; Singh et al. 2011; Jones et al. 2015).

The limited number of morphologically and molecularly well-documented obligate marine fungi that are preserved in herbarium and/or in axenic cultures as reference collections has also been

hampering the classification of newly reported fungal species into obligate or facultative marine fungi (Rämä et al. 2014).
Other terrestrial-like fungi morphologically different from facultative marine fungi have been frequently reported from deep-sea sediments and from other offshore substrates (Morrison-Gardiner 2002; Damare et al. 2006; Burgaud et al. 2009; Nagano et al. 2010; Singh et al. 2011, 2012; Sakayaroj et al. 2012). Some of these fungi have also been recorded in hypersaline solar salterns (Nayak et al. 2012). The close relationship with terrestrial taxa and the uncertainly of whether these fungi were metabolically active in marine ecosystems prompted some authors to adopt a more generic term to designate these fungi, such as marine-derived or ubiquitous fungi (Burgaud et al. 2009; Jones 2011a; Overy et al. 2014a). These terms encompasse mostly mitosporic fungi included in genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma* and *Trichoderma* (Morrison-Gardiner 2002; Damare et al. 2006; Burgaud et al. 2009; Nagano et al. 2010; Singh et al. 2011, 2012; Sakayaroj et al. 2012; Overy et al. 2014a).
Nevertheless, some facultative marine fungi and "marine-derived fungal species" were demonstrated by laboratory simulation experiments or molecular analysis to be dominant and metabolically active in marine environments, particularly in deep-sea habitats; these fungi have been hypothesized to play a much greater role in these ecosystems than truly marine fungi (i.e. obligate marine fungi or marine fungi *sensu strictu*) as a consequence of their physiological and metabolic versatility in response to different ecological conditions (Raghukumar and Raghukumar 1998; Raghukumar and Raghukumar 1999; Damare et al. 2006; Burgaud et al. 2009, 2013; Huang et al. 2011; Singh et al. 2011, 2012). Facultative marine fungi and "marine-derived fungi" have been postulated to reach deep oceanic habitats in the form of spores transported by wind or fungal inocula attached to vegetal substrates and/or particulate organic matter (Damare et al. 2006; Singh et al. 2011). Osmo- and halotolerance of these fungi have been explained as result of a long-term evolution process (Damare et al. 2006; Huang et al. 2011).
The recent recognition of marine environments as potential hot spots for chemically new secondary metabolites produced by marine fungi (mostly "marine-derived fungi") has promoted the increase of studies in these environments with biotechnological purposes, in both mycological and chemical research fields (Jones 2011a; Overy et al. 2014a). However, the lack of consensus in the terminology used to classify the fungi has contributed to different classifications of the same or new species based on personal interpretation of the terms.
In an attempt to uniformise the terminology, Overy et al. (2014a) and Jones et al. (2015) argued that truly marine fungi may be distinguished from terrestrial counterparts based on their ecological roles; these fungi are functionally active in marine ecosystems. Jones et al. (2015) referred also that the frequency of occurrence of fungi on marine ecosystems might be used as a criterion to distinguish marine from terrestrial fungi.

For decades, marine fungi have been classified based exclusively on the morphology of their fruiting structures combined with geographical distribution, host spectrum and asexual morphs

(Kohlmeyer and Kohlmeyer 1979; Kohlmeyer and Volkmann-Kohlmeyer 1991; Hyde et al. 2000; Jones et al. 2009). For some taxonomic groups, the ultrastructure and ontogeny of spore appendages and sheaths were also considered in the classification process (Jones and Moss 1978; Pang 2002; Jones 2011a).

However, with the advent of molecular techniques and particularly DNA sequence analysis (e.g. nuclear ribosomal genes), morphological features were demonstrated not to be efficient in delineating some genera or distinguish species (Campbell et al. 2005; Pang and Mitchell 2005; Aveskamp et al. 2010; Sakayaroj et al. 2011), such as cryptic species (Jones 2011a, b).

Moreover, molecular methods revealed that the majority of evolutionary reconstructions based on morphological characters, nutritional modes and ecologies were unrealistic. As pointed out by Nagahama (2006), sequence-based identification process provides scalable genetic distances that enable a better interpretation of phylogenetic relationships between fungal species. Also, some characters classically used in taxonomy and systematics, such as spore appendages, ascus dehiscence and hamathecium structures, have been demonstrated to be homoplastic (Spatafora et al. 1998; Schoch et al. 2009a; Zhang et al. 2009; Jones 2011b; Sakayaroj et al. 2011); within the two most representative classes of Ascomycota (Dothideomycetes and some Sordariomycetes), morphological characters may either represent retained ancestral or new traits, as a consequence of a convergent or parallel evolution to adapt to similar environmental conditions and selection pressures (Spatafora et al. 1998; Schoch et al. 2009a, b; Zhang et al. 2009; Sakayaroj et al. 2011). This finding has been hampering the construction of taxonomic keys able to distinguish phylogenetic groups based on morphological characteristics.

Because some genes were demonstrated to be highly conserved, a combined use of multiple genes in a multilocus sequence typing approach has proved to be more phylogenetically informative and can resolve different taxonomic issues (Campbell et al. 2003, 2005; Schoch et al. 2009a, b; Suetrong et al. 2009; Aveskamp et al. 2010; Sakayaroj et al. 2011; Jones et al. 2012; Pang 2012).

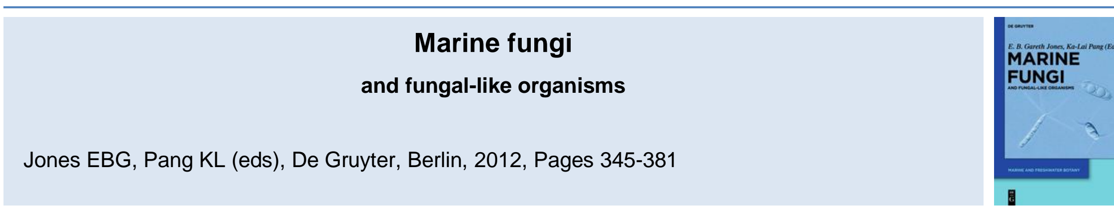
Apart from clarifying phylogenetic relationships between morphological similar species, molecular methods also have been contributed to the understanding of the origin of marine fungi. Higher marine fungi were demonstrated to have a polyphyletic origin (Kohlmeyer and Kohlmeyer 1979). More recent molecular phylogenetic data revealed that deep-branching fungal sequences were found more frequently in terrestrial than marine environments (Richards et al. 2012). These findings suggested that principal lineages of marine fungi were derived from terrestrial ancestors; multiple and independent transitions from terrestrial to marine environment had occurred along the evolutionary scale (Spatafora et al. 1998; Hyde et al. 2000; Schoch et al. 2009a, b; Jones and Pang 2012; Richards et al. 2012; Overy et al. 2014a). Schoch et al. (2009b) also hypothesized that all ascomycetous fungi were derived from a saprobic/non-lichenised ancestor, producer of apothecioid ascomata. These transitions that might have occurred gradually from terrestrial to freshwater and then to marine environments were

accompanied by further morphological adaptations in fungal structures and in the spore-dispersion strategy (Vijaykrishna et al. 2006; Sakayaroj et al. 2011; Jones and Pang 2012).
The identification based on DNA sequences has enabled also the discovery of many novel lineages of unculturable and/or non-fruiting fungi (Pang and Mitchell 2005; Jones 2011a, b; Richards et al. 2012) and links between sexual and asexual morphs (Aveskamp et al. 2010; Abdel-Wahab and Bahkali 2012; Wijayawardene et al. 2012; Jones et al. 2015), contributing to a more realistic and accurate estimate of total diversity of fungi in marine environments. Since 2011, the "one fungus, one name" system was approved, ending with the system of dual nomenclature applied to pleomorphic fungal species (Wijayawardene et al. 2012; Hibbett and Taylor 2013).
Even though a large number of marine species have been already sequenced, some of these species could not be assigned to any taxonomic position given the low representativeness of their gene sequences in public databases and absence of phylogenetically related fungi (Jones et al. 2009, 2012; Jones and Pang 2012). The isolation and sequencing of all described fungi is thus fundamental to confirm their morphology-based taxonomic placement (Suetrong et al. 2009; Jones 2011a; Jones et al. 2012, 2015; Pang 2012) and taxonomic placement of related species as well. Many marine fungi, in particular members of the Dothideomycetes, await assignment to a family or order (Schoch et al. 2009a).
Finally, molecular methods have contributed for a better understanding of ecology, functional role and geographical distribution of already described species (Pang and Mitchell 2005; Richards et al. 2012).

Currently, 1,112 marine fungal species have been reported from marine environments (Jones et al. 2015). This very recent estimate includes truly or "marine-derived fungi" (yeasts and filamentous fungi) and other basal fungal lineages (e.g. Blastocladiomycota and Chytridiomycota).
Jones (2011b) estimated 10,000 fungal species in marine environments if marine-derived, cryptic and unculturable filamentous fungal species and yeasts were considered. Although this number might be overestimated (Overy et al. 2014a), the increase of survey effort or examination of new substrates (e.g. seaweeds, intertidal plants, coral reefs, sediments, water), new habitats (e.g. deep-sea environments) and new geographical locations (e.g. Africa, South America and Arctic regions), will certainly contributes to a significant rise of fungal diversity (Jones 2011a, b; Jones et al. 2015; Alias et al. 2010; Suetrong et al. 2009; Pang and Jones 2012).
According to Jones et al. (2015), only the total documentation of all fungal and fungal-like species in marine environments enable the entire understanding of phylogenetic relationships between species and taxonomic identity and ecology of each species.

1.2 Salt marsh fungi

Calado ML and Barata M



Marine fungi

and fungal-like organisms

Jones EBG, Pang KL (eds), De Gruyter, Berlin, 2012, Pages 345-381

Introduction: Salt marsh ecosystem functioning and the importance of the microbial community

Salt marsh ecosystem

Salt marshes represent coastal marine ecosystems that occur mainly in temperate and high-latitude estuaries (Allen and Pye 1992; Simas et al. 2001) and are exposed to low hydrodynamic conditions and periodic tidal flooding (Simas et al. 2001). They are plastic, dynamic systems created by the combined action of water, sediments and vegetation, and constitute a typical example of open ecosystems (Chapman 1977; Boorman 1999). Salt marshes have long been recognized as being one of the most productive ecosystems in the world (Kohlmeyer and Kohlmeyer 1979; McLusky and Elliott 2004) due to their high primary production rates (Bouchard and Lefeuvre 2000; McLusky and Elliott 2004). A number of emergent macrophytes, in particular *Spartina* spp., *Juncus roemerianus* and *Phragmites australis*, are grass-like plants that thrive in such an environment and represent one of the main sources of nutrients and organic matter (Teal 1962; Christian et al. 1990; Newell et al. 1996b; Van Ryckegem et al. 2006). The primary production of these macrophytes is essentially composed of highly refractory lignocellulosic compounds, such as lignin, hemicellulose and cellulose (Maccubbin and Hodson 1980; Benner et al. 1984a, b; Torzilli and Andrykovitch 1986; Newell et al. 1996b; Lyons et al. 2010), and hence only a small fraction is consumed as living tissue (Teal 1962; Maccubbin and Hodson 1980); most of the production is actually converted into detritus, which either remains in the salt marsh or is transported to coastal waters (Teal 1962; Asaeda et al. 2002). For these emergent macrophytes, the decay process is initiated in the standing crops, and continues after abscission and deposition of dead plant material onto the marsh surface (Fell and Hunter 1979; Newell and Fallon 1989; Newell et al. 1989, 1998; Christian et al. 1990; Samiaji and Barlocher 1996; Gessner 2001; Van Ryckegem et al. 2006; Menéndez and Sanmartí 2007). Much of the decay of marsh grass tissue takes place above the sediment (Newell and Porter 2000).

Decomposer microbial community

The decomposer microbial community, including fungi and bacteria, assumes a fundamental ecological role in the degradation of plant material, which is enriched with structural polymers, and in the consequent release of nutrients that are essential to the metabolism of a wide marine community (Benner et al. 1984b; Boorman 1999; Newell and Porter 2000; Lyons et al. 2005). Though the role of fungi in this process has been long neglected, several studies have highlighted the importance of the metabolic activities of these saprobic microorganisms on the biogeochemical carbon and nutrients cycles, and in the energy fluxes within these ecotonal marine ecosystems (Gessner and Goos 1973; Torzilli and Andrykovitch 1986; Newell 1996; Newell et al. 1996b; Hyde et al. 1998; Gessner et al. 2007).

Saprobic fungi that colonize standing-dead tissues of salt marsh grasses initiate the decay process (Torzilli and Andrykovitch 1986; Samiaji and Barlocher 1996; Lyons et al. 2005), and represent the main secondary producers of the microbial community (Newell and Fallon 1989; Newell et al. 1989, 1996a, b, 2000a; Newell 1996, 2001a; Castro and Freitas 2000; Gessner 2001; Findlay et al. 2002; Van Ryckegem et al. 2006, 2007). Bacteria may become more active in the latter phase of decomposition (i.e., when the plant material collapses onto the marsh sediment surface) (Benner et al. 1984b; Newell et al. 1989; Newell and Porter 2000). However, Buchan et al. (2003) and Lyons et al. (2005) demonstrated that metabolically-active bacteria and fungi co-occur on *Spartina* detritus, which contradicts this idea of temporally segregated interventions during the decay process, but apparently without establishing species-specific ecological associations.

In the microbial community associated with standing-decaying tissues of emergent macrophytes, there is a clear dominance of fungi over bacteria. This is expressed in biomass and productivity. This dominance occurs because the morphological and physiological characteristics of the saprobic fungi confer an adaptive advantage on the use and degradation of this substrate. In fact, in addition to their ability to tolerate a wide range of environmental conditions, fungi can degrade the most resistant substrates in a more efficient manner than bacteria. Filamentous fungi are well suited to penetrate substrates with their rigid cell walls, apical growth and ability to produce lignocellulose-degrading enzymes (Torzilli 1982; Newell 1996; Newell et al. 1996b; Raghukumar 2004b). Saprobic fungi act on the surface or within the tissues of macrophytes by the production of lignocellulolytic enzymes and physical penetration of the host cell walls, bringing about decomposition of senescent tissues (Newell and Porter 2000). Additionally, saprobic fungi have the ability to retain and convert inorganic nitrogen into fungal biomass during the initial phases of plant tissue decomposition (Findlay et al. 2002; Van Ryckegem et al. 2006, 2007) and immobilize this nutrient from the surrounding environment (Newell 1996; Van Ryckegem et al. 2006). The incorporation of nitrogen into fungal biomass, together with the extracellular enzymes produced during the process, results in a nutritive enrichment of substrates, which in turn becomes more palatable to several animal consumers (Raghukumar 2004b). The fungal community associated with the decomposition of macrophytes

is composed mainly of ascomycetes (Gessner and Kohlmeyer 1976; Newell et al. 1996a, 2000a; Newell 2001a, b; Barata 2002; Buchan et al. 2003; Van Ryckegem and Verbeken 2005a, b, c).

Mycota of salt marshes: biotic and abiotic factors affecting community structure

Despite similar general biophysical characteristics, salt marsh ecosystems present some environmental and ecological variations that will reflect on the composition and dynamics of the fungal community. The marine fungal community in salt marshes, as in other ecosystems, is composed of ubiquitous species, which occur on a broad range of substrates and environmental conditions, and also by other species that appear to be strictly associated with particular ecological niches (Gessner and Kohlmeyer 1976). The presence of a given fungus in the ecosystem depends on an appropriate combination of various biotic and abiotic factors, which vary according to species. These diverse factors include:

(i) degree of host/substrate specificity (Apinis and Chesters 1964; Newell and Porter 2000; Blum et al. 2004; Torzilli et al. 2006; Lyons et al. 2010);

(ii) ability to interact and compete with other microorganisms (Torzilli and Andrykovitch 1986; Buchan et al. 2003; Lyons et al. 2005);

(iii) vulnerability/resistance to predation (Newell and Wasowski 1995; Newell 2001a, b); and

(iv) ecological requirements, such as water (Newell et al. 1996a; Poon and Hyde 1998a) and oxygen availability (Wong and Hyde 2002; Menéndez and Sanmartí 2007), dissolved organic nutrients (Newell et al. 1996a, 2000a; Newell and Porter 2000; Newell 2001b), salinity (Van Ryckegem and Verbeken 2005c), and temperature (Castro and Freitas 2000; Van Ryckegem et al. 2007).

Host/substrate specificity

Among the intrinsic biological and environmental factors mentioned, the host/substrate specificity - which is related to the chemical and structural composition of plant tissues - appears to be primarily responsible for determining fungal community composition and productivity (Fell and Hunter 1979; Newell and Porter 2000; Newell et al. 2000a; Blum et al. 2004; Torzilli et al. 2006; Van Ryckegem et al. 2006, 2007; Lyons et al. 2010). This specificity occurs during the selection process of the host plant species to be colonized, but also in the choice of the plant tissue.

Host plant and associated fungal diversity

Studies of salt marsh fungi associated with diverse host plants reveal no overlap between the fungal-decay communities, which emphasizes the general high-level specificity with the chemical and structural characteristics of each plant (Newell and Porter 2000; Blum et al. 2004; Torzilli et al. 2006). Torzilli et al. (2006) compared the mycota associated with four salt marsh plants — *S. alterniflora*, *J. roemerianus*, *Distichlis spicata* and *Sarcocornia perennis* — and concluded that the greater the similarity between the type of plant tissues, the greater is the similarity between the associated fungal communities. The same conclusion was reached by Lyons et al. (2010), who found the same major ascomycetes on various species of *Spartina* (*S. alterniflora*, *S. foliosa*, *S. alterniflora* x *S. foliosa*, *S. densiflora*).

Walker and Campbell (2010) inventoried the fungal community associated with *S. alterniflora* and *J. roemerianus* using morphological and molecular approaches, and obtained different results. The morphological analyses revealed different species on host plants, but terminal-restriction fragment length polymorphism community profiles showed that more than 50% of the fungal terminal-restriction fragments were found on both plants. The authors suggested that the absence of fruiting structures of the same species on *S. alterniflora* and *J. roemerianus* might indicate that some fungi are able to colonize but not sporulate on both hosts, and thus might be host-specific to complete their lifecycle.

A comparison of species composition of fungal communities associated with the main primary producers in marsh ecosystems (Table 1) confirms the above observation.

Table 1 Filamentous fungi associated with *Juncus roemerianus*, *Spartina* spp. and *Phragmites australis* in marsh ecosystems

Fungi	Host Plant		
	Juncus roemerianus	*Spartina* spp.	*Phragmites australis*
Ascomycota			
Amauroascus albicans (Apinis) Arx		Barata (2002)	
Amphisphaeria culmicola Sacc.		Barata (2002)	
Aniptodera chesapeakensis Shearer *et* M.A. Mill. *		Kohlmeyer and Volkmann-Kohlmeyer (2002)	Poon and Hyde (1998a)
Aniptodera juncicola Volkm.-Kohlm. *et* Kohlm. *	Volkmann-Kohlmeyer and Kohlmeyer (1994); Jones (2011a)		
Anthostomella atroalba Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1998b); Jones (2011a)		
Anthostomella poecila Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.*	Kohlmeyer et al. (1995b); Walker and Campbell (2010)		
Anthostomella punctulata (Roberge ex Desm.) Sacc.			Van Ryckegem et al. (2007)
Anthostomella semitecta Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1995b); Jones (2011a)		
Anthostomella spissitecta Kohlm. *et* Volkm.-Kohlm.*		Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Anthostomella torosa Kohlm. *et* Volkm.-Kohlm.*	Kohlmeyer and Volkmann-Kohlmeyer (2002); Jones (2011a)		
Anthostomella sp.		Barata (2002)	
Apiospora montagnei Sacc.			Van Ryckegem and Verbeken (2005a)
Aposphaeria sp.			Van Ryckegem and Verbeken (2005a,b)
Aquamarina speciosa Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.*	Kohlmeyer et al. (1995d); Jones (2011a)		
Aropsiclus junci (Kohlm. *et* Volkm.-Kohlm.) Kohlm. *et* Volkm.-Kohlm.*	Kohlmeyer and Volkmann-Kohlmeyer (1994); Jones (2011a)		
Atkinsonella hypoxylon (Peck) Diehl		Barata (2002)	
Atrotorquata lineata Kohlm. *et* Volkm.-Kohlm.*	Kohlmeyer and Volkmann-Kohlmeyer (1993b); Jones (2011a)		
Belonium heteromorphum (Ellis *et* Everh.) Seaver		Barata (2002)	
Botryosphaeria festucae (Lib.) Arx *et* E. Müll.			Van Ryckegem and Verbeken (2005a,b)
Brunnipila palearum (Desm.) Baral		Barata (2002)	
Buergenerula spartinae Kohlm. *et* R.V. Gessner *		Barata (2002); Buchan et al. (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); ; Buchan et al. (2003); Walker and Campbell (2010)	
Byssothecium obiones (P. Crouan & H. Crouan) M.E. Barr *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Ceratosphaeria sp.	Fell and Hunter (1979)		
Ceriosporopsis halima Linder *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Chaetomium crispatum (Fuckel) Fuckel		Barata (2002)	
Chaetomium funicola Cooke		Barata (2002)	
Chaetomium globosum Kunze		Barata (2002)	Poon and Hyde (1998a)
Chaetomium thermophilum La Touche		Barata (2002)	

Chaetomium sp.	Fell and Hunter (1979)		
Cistella fugiens (W. Phillips) Matheis			Van Ryckegem and Verbeken (2005b)
Claviceps purpurea (Fr.) Tul.		Barata (2002)	
Claviceps sp.		Barata (2002)	
Corollospora maritima Werderm. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Corynascus sepedonium (C.W. Emmons) Arx		Barata (2002)	
Decorospora gaudefroyi (Pat.) Inderb., Kohlm. *et* Volkm.-Kohlm. *		Barata (2002)	
Didymella glacialis Rehm			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Didymella sp.		Barata (2002)	Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Didymosphaeria lignomaris Strongman *et* J.D. Mill. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Discostroma sp.			Van Ryckegem and Verbeken (2005a)
Ellisiodothis inquinans (Ellis *et* Everh.) Theiss.		Barata (2002)	
Gaeumannomyces graminis var. *graminis* (Sacc.) Arx *et* D.L. Olivier		Buchan et al. (2003)	
Gaeumannomyces medullaris Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.(anamorph *Trichocladium medullare* Kohlm. *et* Volkm.-Kohlm.) *	Kohlmeyer et al. (1995c); Jones (2011a)		
Gaeumannomyces sp.			Poon and Hyde (1998a)
Gibberella gordonii C. Booth		Barata (2002)	
Gibberella zeae (Schwein.) Petch			Van Ryckegem and Verbeken (2005a,b)
Gibberella sp.		Barata (2002)	
Gloeotinia granigena (Quél.) T. Schumach.	Walker and Campbell (2010)		
Glomerobolus gelineus Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (1996a); Jones (2011a)		
Gnomonia salina E.B.G. Jones *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Guignardia sp.	Fell and Hunter (1979)		
Haematonectria haematococca (Berk. *et* Broome) Samuels *et* Rossman			Poon and Hyde (1998a)
Haligena elaterophora Kohlm. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Halosarpheia culmiperda Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1995c); Jones (2011a)		
Halosarpheia phragmiticola Poon *et* K.D. Hyde *			Poon and Hyde (1998a)
Heleiosa barbatula Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1996); Jones (2011a)		
Helicascus kanaloanus Kohlm. *		Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Hydropisphaera arenula (Berk. *et* Broome) Rossman *et* Samuels			Van Ryckegem and Verbeken (2005a)
Hydropisphaera erubescens (Roberge ex Desm.) Rossman *et* Samuels		Buchan et al. (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); Buchan et al. (2003)	
Julella herbatilis Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1997); Jones (2011a)		

Juncigena adarca Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. (anamorph *Cirrenalia adarca* Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.) *	Kohlmeyer et al. (1997); Jones (2011a)		
Kananascus sp.		Buchan et al. (2003)	
Keissleriella rara Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1995d); Jones (2011a)		
Keissleriella sp.	Fell and Hunter (1979)		
Lachnum spartinae S.A. Cantrell		Kohlmeyer and Volkmann-Kohlmeyer (2002); Buchan et al. (2003)	
Lautospora simillima Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1995a); Jones (2011a)		
Lentithecium arundinaceum (Sowerby) K.D. Hyde, J. Fourn. *et* Yin. Zhang		Barata (2002)	Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Lentithecium fluviatile (Aptroot *et* Van Ryck.) K.D. Hyde, J. Fourn. *et* Yin. Zhang			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Lentithecium lineare (E. Müll. ex Dennis) K.D. Hyde, J. Fourn. *et* Yin. Zhang			Van Ryckegem and Verbeken (2005b)
Leptosphaeria albopunctata (Westend.) Sacc.		Barata (2002)	
Leptosphaeria australiensis (Cribb *et* J.W. Cribb) G.C. Hughes *	Fell and Hunter (1979)	Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Leptosphaeria lacustris (Fuckel) Wint.		Barata (2002)	
Leptosphaeria marina Ellis *et* Everh.*		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Leptosphaeria orae-maris Linder*		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Leptosphaeria pelagica E.B.G. Jones *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); Walker and Campbell (2010)	
Leptosphaeria sp.	Walker and Campbell (2010)		Poon and Hyde (1998a)
Lewia infectoria (Fuckel) M.E. Barr *et* E.G. Simmons			Van Ryckegem and Verbeken (2005a)
Lignincola laevis Höhnk *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	Poon and Hyde (1998a)
Lophiostoma arundinis (Pers.) Ces. *et* De Not.			Van Ryckegem and Verbeken (2005b)
Lophiostoma semiliberum (Desm.) Ces. *et* De Not.			Van Ryckegem and Verbeken (2005b)
Lophodermium arundinaceum (Schrad.) Chevall.			Van Ryckegem and Verbeken (2005a,b)
Loratospora aestuarii Kohlm. *et* Volkm.-Kohlm. *	Kohlmeyer and Volkmann-Kohlmeyer (1993b); Jones (2011a)		
Lulworthia floridana Meyers *		Barata (2002)	
Lulworthia medusa (Ellis *et* Everh.) Cribb *et* J.W. Cribb *		Jones (1963); Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Lulworthia spp.		Barata (2002)	
Magnisphaera spartinae (E.B.G. Jones) J. Campb., J.L. Anderson *et* Shearer *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	Van Ryckegem and Verbeken (2005b)
Massariella sp.		Barata (2002)	
Massarina carolinensis Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1995d); Jones (2011a)		
Massarina phragmiticola Poon *et* K.D. Hyde *			Poon and Hyde (1998a)
Massarina ricifera Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1995c); Walker and Campbell		

	(2010); Jones (2011a)		
Massarina spp.	Fell and Hunter (1979)		Van Ryckegem and Verbeken (2005a)
Massariosphaeria erucacea Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1996); Jones (2011a)		
Massariosphaeria scirpina (G. Winter) Leuchtm.		Barata (2002)	
Massariosphaeria typhicola (P. Karst.) Leuchtm. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Massariosphaeria sp.			Van Ryckegem and Verbeken (2005b)
Meliola spartinae (Ellis *et* Everh.) Berl. *et* Voglino		Barata (2002)	
Micronectriella agropyri Apinis *et* Chesters		Barata (2002)	
Microthecium levitum Udagawa *et* Cain		Barata (2002)	
Microthyrium microscopicum Desm.		Barata (2002)	
Mollisia atriella Cooke		Barata (2002)	
Mollisia cf. *palustris* (Roberge ex Desm.) P. Karst.			Van Ryckegem and Verbeken (2005a)
Mollisia hydrophila (P. Karst.) Sacc.			Van Ryckegem and Verbeken (2005a)
Mollisia retincola (Rabenh.) P. Karst.			Van Ryckegem and Verbeken (2005a,b)
Morenoina phragmitis J.P. Ellis			Van Ryckegem and Verbeken (2005a,b)
Mycosphaerella eurypotami Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1999); Jones (2011a)		
Mycosphaerella lineolata (Roberge ex Desm.) J. Schröt.			Van Ryckegem and Verbeken (2005a,b)
Mycosphaerella salicorniae (Rabenh.) Lindau *		Barata (2002)	
Mycosphaerella spp.	Fell and Hunter (1979); Walker and Campbell (2010)	Barata (2002); Buchan et al. (2002); Buchan et al. (2003); Lyons et al.(2010); Walker and Campbell (2010)	
Naïs inornata Kohlm. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Natantispora retorquens (Shearer *et* J.L. Crane) J. Campb., J.L. Anderson *et* Shearer *		Barata (2002)	
Nectria sp.	Fell and Hunter (1979)		
Ommatomyces coronatus Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1995c); Jones (2011a)		
Orbilia junci Kohlm., Baral *et* Volkm.-Kohlm. (anamorph *Dwayaangam junci* Kohlm., Baral *et* Volkm.-Kohlm.)	Kohlmeyer et al. (1998a); Jones (2011a)		
Otthia sp.	Fell and Hunter (1979)		
Panorbis viscosus (I. Schmidt) J. Campb., J.L. Anderson *et* Shearer *		Barata (2002); Buchan et al. (2003)	
Papulosa amerospora Kohlm. *et* Volkm.-Kohlm. *	Kohlmeyer and Volkmann-Kohlmeyer (1993a); Jones (2011a)		
Paraphaeosphaeria apicicola Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. (anamorph *Coniothyrium* sp.)	Kohlmeyer et al. (1999); Jones (2011a)		
Paraphaeosphaeria michotii (Westend.) O.E. Erikss.			Van Ryckegem and Verbeken (2005a)
Paraphaeosphaeria pilleata Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. (anamorph *Coniothyrium* sp.)	Kohlmeyer et al. (1995d); Jones (2011a)		
Phaeosphaeria anchiala Kohlm., Volkm.-Kohlm. *et* K.M. Tsui	Kohlmeyer et al (2005); Jones (2011a)		
Phaeosphaeria caricinella (P. Karst.) O.E. Erikss.		Barata (2002)	

Phaeosphaeria culmorum (Auersw. ex Rehm) Leuchtm.			Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Phaeosphaeria eustoma (Fuckel) L. Holm			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Phaeosphaeria gessneri Shoemaker *et* C.E. Babc. *		Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Phaeosphaeria halima (T.W. Johnson) Shoemaker *et* C.E. Babc. *		Barata (2002); Buchan et al. (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); Buchan et al. (2003); Lyons et al.(2010); Walker and Campbell (2010)	
Phaeosphaeria herpotrichoides (De Not.) L. Holm		Barata (2002)	
Phaeosphaeria juncina (Auersw.) L. Holm	Fell and Hunter (1979)		
Phaeosphaeria luctuosa (Niessl ex Sacc.) Otani *et* Mikawa			Van Ryckegem and Verbeken (2005a,b)
Phaeosphaeria macrosporidium (E.B.G. Jones) Shoemaker *et* C.E. Babc. *		Barata (2002)	
Phaeosphaeria neomaritima (R.V. Gessner *et* Kohlm.) Shoemaker *et* C.E. Babc. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Phaeosphaeria nodorum (E. Müll.) Hedjar.		Buchan et al. (2003)	
Phaeosphaeria olivacea Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1997); Jones (2011a)		
Phaeosphaeria pontiformis (Fuckel) Leuchtm.			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Phaeosphaeria roemeriani Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1998b); Walker and Campbell (2010); Jones (2011a)		
Phaeosphaeria spartinae (Ellis *et* Everh.) Shoemaker *et* C.E. Babc. *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Phaeosphaeria spartinicola Leuchtm. *		Barata (2002); Buchan et al. (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); Buchan et al. (2003); Lyons et al.(2010); Walker and Campbell (2010)	
Phaeosphaeria typharum (Desm.) L. Holm *		Barata (2002)	
Phaeosphaeria vagans (Niessl) O.E. Erikss.			Van Ryckegem and Verbeken (2005a)
Phaeosphaeria spp.			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Phomatospora bellaminuta Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1995b); Jones (2011a)		
Phomatospora berkeleyi Sacc.			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Phomatospora dinemasporium J. Webster		Barata (2002)	Van Ryckegem and Verbeken (2005b)
Phomatospora phragmiticola Poon *et* K.D. Hyde *			Poon and Hyde (1998a)
Phomatospora spp.	Fell and Hunter (1979)		Van Ryckegem and Verbeken (2005a,b)
Phragmitensis marina M.K.M. Wong, Poon *et* K.D. Hyde *			Poon and Hyde (1998a)
Phyllachora cynodontis Niessl		Barata (2002)	
Phyllachora graminis var. *graminis* (Pers.) Fuckel		Barata (2002)	
Phyllachora sylvatica Sacc. *et* Speg.		Barata (2002)	

Physalospora citogerminans Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1995b); Jones (2011a)		
Pleospora abscondita Sacc. *et* Roum.			Van Ryckegem and Verbeken (2005a)
Pleospora herbarum (Pers.) Rabenh.		Barata (2002)	
Pleospora pelagica T.W. Johnson *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); Buchan et al. (2003); Lyons et al.(2010)	
Pleospora spartinae (J. Webster *et* M.T. Lucas) Apinis *et* Chesters *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002); Buchan et al. (2003)	Poon and Hyde (1998a)
Pleospora vagans var. *vagans* Niessl		Barata (2002)	
Preussia funiculata (Preuss) Fuckel		Barata (2002)	
Pseudohalonectria falcata Shearer			Poon and Hyde (1998a)
Pseudohalonectria halophila Kohlm. *et* Volkm.-Kohlm. *	Kohlmeyer et al (2005); Jones (2011a)		
Remispora hamata (Höhnk) Kohlm.	Fell and Hunter (1979)	Barata (2002)	Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Rivilata ius Kohlm., Volkm.-Kohlm. *et* O.E. Erikss.	Kohlmeyer et al. (1998b); Jones (2011a)		
Schizothecium hispidulum (Speg.) N. Lundq.			Van Ryckegem and Verbeken (2005a)
Scirrhia annulata Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1996); Jones (2011a)		
Sordaria fimicola (Roberge ex Desm.) Ces. *et* De Not.		Barata (2002)	
Sphaerulina albispiculata Tubaki *		Barata (2002)	
Sphaerulina orae-maris Linder *		Jones (1963); Barata (2002)	
Sphaerulina sp.	Fell and Hunter (1979)		
Splanchnonema sp.	Fell and Hunter (1979)		
Sporormia sp.	Fell and Hunter (1979)		
Sporormiella intermedia (Auersw.) S.I. Ahmed *et* Cain ex Kobayasi		Barata (2002)	
Stictis sp.			Van Ryckegem and Verbeken (2005a)
Thelebolus crustaceus (Fuckel) Kimbr.		Barata (2002)	
Tremateia halophila Kohlm., Volkm.-Kohlm. *et* O.E. Erikss. *	Kohlmeyer et al. (1995a); Jones (2011a)	Barata (2002)	
Trichodelitschia bisporula (P. Crouan *et* H. Crouan) Munk		Barata (2002)	
Zopfiella latipes (N. Lundq.) Malloch *et* Cain *			Poon and Hyde (1998a)
Basidiomycota			
Halocyphina villosa Kohlm. *et* E. Kohlm. *		Barata (2002)	
Merismodes bresadolae (Grélet) Singer			Van Ryckegem and Verbeken (2005b)
Nia vibrissa R.T. Moore *et* Meyers *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Puccinia magnusiana Körn.			Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Puccinia phragmitis (Schumach.) Körn.			Van Ryckegem et al. (2007)
Puccinia seymouriana Arthur		Barata (2002)	
Puccinia sparganioides Ellis *et* Tracy		Barata (2002)	
Sporobolomyces sp.			Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Tremella spicifera Van Ryck., Van de Put *et* P. Roberts			Van Ryckegem and Verbeken (2005a,b)

Uromyces acuminatus Arthur		Barata (2002)	
Uromyces argutus F. Kern		Barata (2002)	
Anamorphic fungi			
Acremonium sp.	Fell and Hunter (1979)		
Alternaria alternata (Fr.) Keissl.	Fell and Hunter (1979)	Barata (2002)	Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Alternaria maritima G.K. Sutherl.		Barata (2002)	
Arthrinium phaeospermum (Corda) M.B. Ellis		Barata (2002)	Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Arthrinium sp. (state of *Apiospora montagnei*)			Poon and Hyde (1998a)
Arthrinium sp. (state of *Apiospora* sp.)			Poon and Hyde (1998a)
Arthrinium sp.	Fell and Hunter (1979)		
Arthrobotrys sp.			Poon and Hyde (1998a)
Ascochyta cf. *arundinariae* Tassi			Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Ascochyta cf. *leptospora* (Trail) Hara			Van Ryckegem and Verbeken (2005a)
Ascochyta spartinae Trel.		Barata (2002)	
Ascochyta sp.		Barata (2002)	Van Ryckegem and Verbeken (2005a)
Aspergillus nidulans (Eidam) G. Winter		Barata (2002)	
Aspergillus niger Tiegh.	Fell and Hunter (1979)		
Aspergillus ustus (Bainier) Thom *et* Church		Walker and Campbell (2010)	
Aspergillus spp.	Fell and Hunter (1979)	Barata (2002)	
Asteromyces cruciatus Moreau *et* M. Moreau ex Hennebert *		Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Aureobasidium sp.	Fell and Hunter (1979)		
Bactrodesmium atrum M.B. Ellis			Van Ryckegem and Verbeken (2005b)
Botryodiplodia sp.	Fell and Hunter (1979)		
Botrytis cinerea Pers.		Barata (2002)	
Camarosporium feurichii Henn.			Van Ryckegem and Verbeken (2005a)
Camarosporium sp.			Van Ryckegem and Verbeken (2005a,b)
Chaetasbolisia sp.			Poon and Hyde (1998a)
Chaetospermum camelliae Agnihothr.			Poon and Hyde (1998a)
Cirrenalia macrocephala (Kohlm.) Meyers *et* R.T. Moore *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Cirrenalia pseudomacrocephala Kohlm. *	Fell and Hunter (1979)		
Cladosporium algarum Cooke *et* Massee *		Barata (2002)	
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Fell and Hunter (1979)		
Cladosporium sphaerospermum Penz.	Fell and Hunter (1979)		
Cladosporium spp.	Fell and Hunter (1979)	Barata (2002)	Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Cochliobolus hawaiiensis Alcorn	Fell and Hunter (1979)		Poon and Hyde (1998a)
Cochliobolus tuberculatus Sivan.	Fell and Hunter (1979)		
Colletotrichum sp.			Poon and Hyde (1998a)
Coniothyrium spp.	Fell and Hunter (1979)		

Cremasteria cymatilis Meyers *et* R.T. Moore	Fell and Hunter (1979)		
Cumulospora marina I. Schmidt *		Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Curvularia protuberata R.R. Nelson *et* Hodges	Fell and Hunter (1979)		
Curvularia sp.	Fell and Hunter (1979)		
Cytoplacosphaeria phragmiticola Poon *et* K.D. Hyde *			Poon and Hyde (1998a)
Cytoplacosphaeria rimosa Petr.			Van Ryckegem and Verbeken (2005a,b)
Cytoplea sp.			Poon and Hyde (1998a)
Deightoniella roumeguerei (Cavara) Constant.			Van Ryckegem et al. (2007)
Dendrostilbella sp.			Poon and Hyde (1998a)
Dictyosporium oblongum (Fuckel) S. Hughes			Van Ryckegem and Verbeken (2005b); Van Ryckegem et al. (2007)
Dictyosporium pelagicum (Linder) G.C. Hughes ex E.B.G. Jones *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Dictyosporium toruloides (Corda) Guég.		Jones (1963)	
Drechslera sp.	Fell and Hunter (1979)		
Dumortieria sp.	Fell and Hunter (1979)		
Epicoccum nigrum Link	Fell and Hunter (1979)	Barata (2002)	
Flagellospora sp.	Fell and Hunter (1979)		
Floricola striata Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (2000); Jones (2011a)		
Fusarium incarnatum (Desm.) Sacc.		Walker and Campbell (2010)	
Fusarium spp.	Fell and Hunter (1979)		Van Ryckegem and Verbeken (2005a,b)
Geniculosporium sp.	Fell and Hunter (1979)		
Gliocladium sp.	Fell and Hunter (1979)		
Gliomastix spp.	Fell and Hunter (1979)		Poon and Hyde (1998a)
Halenospora varia (Anastasiou) E.B.G. Jones *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Haplobasidion lelebae Sawada ex M.B. Ellis	Fell and Hunter (1979)		
Hendersonia culmicola Sacc.		Barata (2002)	
Hendersonia culmiseda Sacc.			Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Humicola sp.	Fell and Hunter (1979)		
Hymenopsis chlorothrix Kohlm. *et* Volkm.-Kohlm. *	Kohlmeyer and Volkmann-Kohlmeyer (2001a); Jones (2011a)		
Hyphopolynema juncatile Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (1999); Jones (2011a)		
Khuskia oryzae H.J. Huds.	Fell and Hunter (1979)		
Kolletes undulatus Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer et al (2005); Jones (2011a)		
Koorchaloma galateae Kohlm. *et* Volkm.-Kohlm. *	Kohlmeyer and Volkmann-Kohlmeyer (2001b); Jones (2011a)		
Koorchaloma spartinicola V.V. Sarma, S.Y. Newell *et* K.D. Hyde *		Buchan et al. (2003)	
Leptosphaerulina chartarum Cec. Roux	Fell and Hunter (1979)		
Memnoniella echinata (Rivolta) Galloway	Fell and Hunter (1979)		

Microsphaeropsis spp.			Poon and Hyde (1998a); Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Monodictys austrina Tubaki	Fell and Hunter (1979)		
Myrothecium roridum Tode	Fell and Hunter (1979)		
Neottiospora sp.	Fell and Hunter (1979)		
Neottiosporina australiensis B. Sutton *et* Alcorn			Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Paecilomyces sp.	Fell and Hunter (1979)		
Papulaspora halima Anastasiou	Fell and Hunter (1979)		
Penicillium spp.	Fell and Hunter (1979)	Barata (2002)	Poon and Hyde (1998a)
Periconia cookei E.W. Mason *et* M.B. Ellis	Fell and Hunter (1979)		Van Ryckegem et al. (2007)
Periconia digitata (Cooke) Sacc.	Fell and Hunter (1979)		Van Ryckegem and Verbeken (2005a)
Periconia echinochloae (Bat.) M.B. Ellis	Fell and Hunter (1979)		
Periconia minutissima Corda	Fell and Hunter (1979)		Van Ryckegem and Verbeken (2005a)
Periconia sp.	Fell and Hunter (1979)		
Pestalotia planimi Vize		Barata (2002)	
Pestalotia sp.	Fell and Hunter (1979)		
Pestalotiopsis juncestris Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (2001b); Jones (2011a)		
Phaeoseptoria sp.			Van Ryckegem and Verbeken (2005a)
Phaeosiaria sp.			Poon and Hyde (1998a)
Phialophorophoma litoralis Linder *		Barata (2002)	
Phialophorophoma sp.			Van Ryckegem and Verbeken (2005a,b)
Phoma glomerata (Corda) Wollenw. *et* Hochapfel *		Barata (2002)	
Phoma suaedae Jaap *		Barata (2002)	
Phoma spp.	Fell and Hunter (1979)	Barata (2002)	Poon and Hyde (1998a); Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Phomopsis spp.	Fell and Hunter (1979)	Barata (2002)	Poon and Hyde (1998a)
Phyllosticta spartinae Brunaud		Barata (2002)	
Phyllosticta sp.		Barata (2002)	
Piricauda pelagica T. Johnson *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Pithomyces atro-olivaceus (Cooke *et* Harkn.) M.B. Ellis	Fell and Hunter (1979)		
Pithomyces maydicus (Sacc.) M.B. Ellis	Fell and Hunter (1979)		Poon and Hyde (1998a)
Prathoda longissima (Deighton *et* MacGarvie) E.G. Simmons	Fell and Hunter (1979)		
Psammina sp.	Fell and Hunter (1979)		
Pseudorobillarda phragmitis (Cunnell) M. Morelet *			Poon and Hyde (1998a)
Pseudorobillarda sp.		Barata (2002)	
Pseudoseptoria donacis (Pass.) B. Sutton			Van Ryckegem and Verbeken (2005a); Van Ryckegem et al. (2007)
Pycnodallia dupla Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (2001a); Jones (2011a)		
Pyrenochaeta sp.	Fell and Hunter (1979)		
Rhinocladiella spp.	Fell and Hunter (1979)		Poon and Hyde (1998a)

Scolecobasidium arenarium (Nicot) M.B. Ellis *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Scolecobasidium humicola G.L. Barron *et* L.V. Busch	Fell and Hunter (1979)		
Scolecobasidium salinum (G.K. Sutherl.) M.B. Ellis		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Scopulariopsis spp.	Fell and Hunter (1979)		
Selenophoma sp.	Fell and Hunter (1979)		
Septogloeum spartinae (Ellis *et* Everh.) Wollenw. *et* Reinking		Barata (2002)	
Septonema secedens Corda	Fell and Hunter (1979)		
Septoria sp.	Fell and Hunter (1979)		Van Ryckegem and Verbeken (2005a)
Septoriella phragmitis Oudem.			Van Ryckegem and Verbeken (2005a,b)
Septoriella unigalerita Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (2000); Jones (2011a)		
Septoriella sp.			Poon and Hyde (1998a); Van Ryckegem and Verbeken (2005a,b); Van Ryckegem et al. (2007)
Setosphaeria rostrata K.J. Leonard	Fell and Hunter (1979)	Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Spegazzinia tessarthra (Berk. *et* M.A. Curtis) Sacc.	Fell and Hunter (1979)		Poon and Hyde (1998a)
Sphaeronaema sp.	Fell and Hunter (1979)		
Sporothrix sp.	Fell and Hunter (1979)		
Stachybotrys chartarum (Ehrenb.) S. Hughes	Fell and Hunter (1979)		
Stachybotrys kampalensis Hansf.	Fell and Hunter (1979)		
Stachybotrys nephrospora Hansf.	Fell and Hunter (1979)		
Stachybotrys sp.	Fell and Hunter (1979)	Buchan et al. (2003)	Poon and Hyde (1998a)
Stachylidium bicolor Link	Fell and Hunter (1979)		
Stagonospora abundata Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (2000); Jones (2011a)		
Stagonospora cylindrica Gunnell			Van Ryckegem and Verbeken (2005b)
Stagonospora elegans (Berk.) Sacc. *et* Traverso			Van Ryckegem and Verbeken (2005a,b)
Stagonospora spp.	Fell and Hunter (1979)	Barata (2002); Buchan et al. (2003)	Poon and Hyde (1998a); Van Ryckegem and Verbeken (2005a,b)
Stauronema sp.			Poon and Hyde (1998a)
Stemphylium lycopersici (Enjoji) W. Yamam.	Fell and Hunter (1979)		
Stemphylium maritimum T.W. Johnson *		Barata (2002)	
Stemphylium vesicarium (Wallr.) E.G. Simmons	Fell and Hunter (1979)		
Tetranacriella papillata Kohlm. *et* Volkm.-Kohlm.	Kohlmeyer and Volkmann-Kohlmeyer (2001b); Jones (2011a)		
Tetranacrium sp.			Poon and Hyde (1998a)
Tetraplosphaeria tetraploa (Scheuer) Kaz. Tanaka *et* K. Hiray.	Fell and Hunter (1979)		Poon and Hyde (1998a)
Tiarosporella halmyra Kohlm. *et* Volkm.-Kohlm. *	Kohlmeyer and Volkmann-Kohlmeyer (1996b); Jones (2011a)		
Tracyella spartinae (PK.) Tassi		Barata (2002)	
Trichoderma viride Pers.	Fell and Hunter (1979)		
Trichoderma sp.			Poon and Hyde (1998a)

Tubercularia sp.		Buchan et al. (2003)	
Veronaea sp.	Fell and Hunter (1979)		
Virgaria nigra (Link) Nees	Fell and Hunter (1979)		
Xepicula leucotricha (Peck) Nag Raj	Fell and Hunter (1979)		
Zalerion maritima (Linder) Anastasiou *		Barata (2002); Kohlmeyer and Volkmann-Kohlmeyer (2002)	
Zythia spp.	Fell and Hunter (1979)		
Total taxa	**136**	**132**	**109**

* Marine fungi (based on Jones et al. 2009, 2011a)

The list includes all the taxa associated with these plant hosts, mentioned in the following studies: Jones 1963 (3 taxa); Fell and Hunter 1979 (88 taxa); Kohlmeyer and Volkmann-Kohlmeyer 1993a (1 taxa); Kohlmeyer and Volkmann-Kohlmeyer 1993b (2 taxa); Kohlmeyer and Volkmann-Kohlmeyer 1994 (1 taxa); Volkmann-Kohlmeyer and Kohlmeyer 1994 (1 taxa); Kohlmeyer et al 1995a (2 taxa); Kohlmeyer et al 1995b (4 taxa); Kohlmeyer et al 1995c (4 taxa); Kohlmeyer et al 1995d (4 taxa); Kohlmeyer and Volkmann-Kohlmeyer 1996a (1 taxa); Kohlmeyer and Volkmann-Kohlmeyer 1996b (1 taxa); Kohlmeyer et al 1996 (3 taxa); Kohlmeyer et al 1997 (3 taxa); Kohlmeyer et al 1998a (1 taxa); Kohlmeyer et al 1998b (3 taxa); Poon and Hyde 1998a (40 taxa); Kohlmeyer and Volkmann-Kohlmeyer 1999 (1 taxa); Kohlmeyer et al 1999 (2 taxa); Kohlmeyer and Volkmann-Kohlmeyer 2000 (3 taxa); Kohlmeyer and Volkmann-Kohlmeyer 2001a (2 taxa); Kohlmeyer and Volkmann-Kohlmeyer 2001b (3 taxa); Barata 2002 (115 taxa); Buchan et al 2002 (5 taxa); Kohlmeyer and Volkmann-Kohlmeyer 2002 (40 taxa); Buchan et al 2003 (16 taxa); Kohlmeyer et al 2005 (3 taxa); Van Ryckegem and Verbeken 2005a (58 taxa); Van Ryckegem and Verbeken 2005b (40 taxa); Van Ryckegem et al 2007 (27 taxa); Lyons et al 2010 (4 taxa); Walker and Campbell 2010 (12 taxa); Jones 2011a (45 taxa).The names of the taxa follow Index Fungorum (http://www.indexfungorum.org), except Byssothecium obiones.

The overlap of fungal community between all possible host combinations (*Spartina* spp./*J. roemerianus*, *J. romerianus*/*P. australis*, *Spartina* spp./*P. australis* and *Spartina* spp./*J. roemerianus*/*P. australis*), is very low (<5%), which means that each host plant supports a distinct mycota. In fact, from the 332 taxa found on *Spartina* spp., *J. roemerianus* and/or *P. australis*, 89% are exclusively associated with one, 9% associated with two and 2% associated with the three host species, like *Remispora hamata* (doubtful species) and *Alternaria alternata* (Table 1).

Spartina species

Spartina appears to be conducive for the growth of saprobic fungi, given its high lignocellulose content and non-lignin cinnamyl phenols (Newell et al. 1996b). A total of 132 taxa of higher filamentous fungi have been documented from *Spartina* spp. (Table 1). Among the various species associated with this standing marsh grass, *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. II are ubiquitous and dominant in the community, and exhibit high spore-expulsion rates (Newell and Wasowski 1995; Newell and Zakel 2000; Newell 2001a; Buchan et al. 2002, 2003; Lyons et al. 2010). Additional common species in this community are *Phaeosphaeria halima* and *Buergenerula spartinae* (Newell and Zakel 2000; Newell 2001a; Buchan et al. 2002, 2003; Lyons et al. 2010; Walker and Campbell 2010).

Juncus roemerianus

Another salt marsh grass, *J. roemerianus*, supports a surprisingly high number of fungal taxa (Table 1). Most of the fungi associated with this host have been collected and identified by the Kohlmeyers and their colleagues in marshes located on the east coast of the United States. Jones (2011a) has recently updated the list of fungi documented by Kohlmeyer and Volkmann-Kohlmeyer (2001c). One-hundred-thirty-six fungi have been identified on *J. roemerianus* (Table 1). Newell and Porter (2000) have pointed out that *Loratospora aestuarii, Papulosa amerospora, Aropsiclus junci, Anthostomella poecila, Physalospora citogerminans, Scirrhia annulata, Massarina ricifera* and *Trematеia halophila* are the most common fungi occurring on this substrate.

Phragmites australis

P. australis, another host that has been extensively surveyed for fungi, is a cosmopolitan grass that has a widespread worldwide distribution, colonizing not only intertidal marshes but also freshwater and terrestrial habitats in temperate and subtropical regions. Over 300 fungi have been reported for this plant (Wong and Hyde 2001, 2002), of which 109 species were detected in intertidal marshes in Hong Kong (Table 1).

Poon and Hyde (1998a) identified 41 species associated with *P. australis* in an intertidal subtropical marsh in Hong Kong, from which *Massarina phragmiticola*, *Phomatospora phragmiticola* and *Cytoplacosphaeria phragmiticola* were described as new species (Table 1). Wong et al. (1998) described *Phragmitensis marina* from the same locality, while Poon and Hyde (1998a) noted that *Lignincola laevis*, *Trichoderma* sp., *Halosarpheia phragmiticola* and *Colletotrichum* sp. were the most common species on this host.

Host tissue preference

In addition to host-specificity, the majority of the saprobic fungi also exhibit other ecological requirements that determine its occurrence on different parts of the host plant (Newell and Wasowski 1995; Newell et al. 1996b; Newell 2001a; Kohlmeyer and Volkmann-Kohlmeyer 2001c). This pattern may be a result of a nutritive preference for a particular substrate and/or interactions of mutualism, parasitism or competition that are established among the different species of fungi.

P. spartinicola and *Mycosphaerella* sp. II, for example, occur preferably on the leaf blades of *Spartina* spp. and are involved in lignocellulose degradation (Bergbauer and Newell 1992; Newell et al. 1996b; Newell and Porter 2000); both species seem to establish a highly efficient mutualistic relationship that suppresses potential competitors (Newell and Porter 2000; Newell 2001a; Buchan et al. 2003). *B. spartinae* is dominant on leaf sheaths of *Spartina* spp., occurring

in non-melanized patches (Newell 2001a), but it can also colonize the leaf blades, developing characteristic melanotic patches (Buchan et al. 2002). These findings support the hypothesis of Newell and Porter (2000) that the melanization of leaf blades by *B. spartinae* is the result of competition between this species and the complex of *P. spartinicola* and *Mycosphaerella* sp. II.

In a given host plant, the colonization of the diverse plant tissues by saprobic fungi may or may not proceed simultaneously. Van Ryckegem and Verbeken (2005b) have observed that fungal sexual-reproductive structures in *P. australis* stems only appeared after three months of senescence, when nearly 50% of the leaf sheath tissue was decomposed. This time lag in the colonization process was attributed by the fact that the stems were more recalcitrant and consequently less susceptible to fungal breakdown than the sheaths (i.e., fewer stomata, more sclerenchymatous tissue and thicker cuticle).

Species composition of the fungal community associated with a particular plant tissue does not generally remain unchanged, since the plant material undergoes physical and chemical changes during decomposition and different fungi require specific nutrients for their metabolism. Fell and Hunter (1979) directly compared the fungal communities colonizing *J. roemerianus* leaves of different physiological states and distinguished fungi that occurred mainly or exclusively in living, senescent or dead-standing leaves. Buchan et al. (2002, 2003) reached the same conclusions, having found fungi present in early- or late-decay blades of S. alterniflora and other species in both decay stages. Van Ryckegem and Verbeken (2005b) argued that the reduction of carbon resources may lead to an overlap of ecological niches, thereby contributing to an increase in competition between species; in this context, the species with higher antibiotic activity and with a broader spectrum of enzymes would certainly be favoured.

Thus, in a particular decaying plant tissue, one can frequently detect patterns in the succession of fungi, with total or partial replacement of the colonizing species (Kohlmeyer and Volkmann-Kohlmeyer 2001c; Buchan et al. 2002, 2003; Van Ryckegem and Verbeken 2005a, b; Van Ryckegem et al. 2007). Van Ryckegem and Verbeken (2005a) followed the decay and simultaneous colonization process of the leaf sheaths of *P. australis* by saprobic fungi, and characterized three successive phases:

(i) The process begins with a pioneer community composed of few species, like *Sporobolomyces* sp., *Alternaria alternata*, *Cladosporium* sp., *Septoriella* sp., *Phoma* sp. and *Phaeosphaeria* sp. (weak pathogens, biotrophic species and opportunistic saprotrophs). These exhibit a low tolerance to stress.

(ii) The second phase includes a more closed and mature community, with a high diversity of more competitive species, such as *Stictis* sp. and *Lophodermium arundinaceum*.

(iii) The third phase presents an impoverished community dominated by few stress-tolerant species and/or highly competitive taxa, like *Lentithecium arundinaceum* and *Mycosphaerella lineolata*.

The presence of dominant fungi in plant material, however, seems to be independent of the degree of decay of plant tissue (Buchan et al. 2002), which apparently only has effects on the spore-expulsion rate (Newell 2001a; Buchan et al. 2003).
In the latter stages of decomposition, the dead plant tissues finally detach and reach the marsh sediment surface, which offers different microenvironmental conditions. Under these new abiotic conditions, a shift in species composition of the fungal community occurs with an alteration of the productivity of the community (Newell et al. 1989; Kohlmeyer and Volkmann-Kohlmeyer 2001c; Van Ryckegem and Verbeken 2005a, b; Van Ryckegem et al. 2006, 2007).

Ecological requirements

Tidal regime

In marsh ecosystems fungi usually display patterns of vertical distribution on the colonized emergent macrophytes, which go beyond their nutritive requirements and ability to compete with other fungi but also reflect their tolerance limits to environmental conditions, particularly tidal flooding (Gessner 1977; Barata 1997, 2002; Poon and Hyde 1998b; Kohlmeyer and Volkmann-Kohlmeyer 2001c; Van Ryckegem and Verbeken 2005a, b, c; Van Ryckegem et al. 2006, 2007). Vertical distribution of fungi may distinguish between obligate and facultative marine species. Gessner (1977), Kohlmeyer and Volkmann-Kohlmeyer (2001c) and Barata (2002) characterized the mycota associated with *S. alterniflora*, *S. maritima* and *J. roemerianus*. They found that in general terrestrial halotolerant fungi occurred at the tips of the leaves, which rarely or never become submerged. Obligate marine fungi occupy the lower periodically-inundated portions of the leaves, and facultative marine fungi overlap both plant portions.

Seasonality

Environmental fluctuations inherent to seasonality do not seem to interfere with the species composition of fungal communities associated with a particular marsh grass (Torzilli et al. 2006). Nevertheless, seasonality has significant impact on the differentiation of reproductive structures (Newell 2001a) and the abundance of several species (Buchan et al. 2003), as well as on the biomass and productivity of the entire fungal community (Samiaji and Barlocher 1996; Castro and Freitas 2000; Newell and Porter 2000; Newell 2001b).
Even though Castro and Freitas (2000) and Newell (2001b) have shown that fungal biomass and productivity increased during winter and spring and diminished during the summer and fall periods, the potential reasons for these phenomena were different. Castro and Freitas (2000) regarded the decrease of fungal biomass during summer with higher air temperatures and salinity resulted in less favorable moisture conditions for fungal survival. Contrarily, Newell (2001b) suggested that the higher tides and higher rainfall that occurred in the late summer and fall during that particular study, could have contributed to a reduction of fungal biomass and

productivity because the leaves had become more accessible to mycophagous invertebrates and/or bacterial competitors, and had lost more nutrients through leaching.

Nitrogen

The availability of nitrogen in plant tissues and the surrounding environment is likely to be one of the key factors that regulate decomposer activity (Torzilli and Andrykovitch 1986) and fungal productivity (Newell et al. 1996a, 2000a; Newell 2001b). In fact, the studies previously mentioned have demonstrated that when nitrogen increases, fungal productivity also increases; this observation seems to suggest that nitrogen might be the limiting factor in these ecosystems.

Water supply

Newell et al. (1996a) have demonstrated that the fungal community is more productive in a normal regime of repeated drying/wetting episodes, being incapable of achieving higher production when water supply is constant, which proves that this community is well adapted to these environments. On the other hand, Poon and Hyde (1998b) and Wong and Hyde (2002) compared the fungal community associated with *P. australis* in two intertidal areas subjected to distinct water-availability regimes, and observed that fungal diversity was higher under periodic submersion than under a permanent one. Barata (2006) also confirmed this finding in a study performed with *S. maritima* baits in two zones of a salt marsh that were exposed to different periods of submergence; the intertidal zone was the most favourable place for colonization by a high diversity of marine fungi. Newell (1995) added that when the plant tissues become dry during a dry season, the colonizing fungi can interrupt their metabolic activity and resume when humid conditions are restored, without any loss of biomass.

Pollution

The impact of anthropogenic pollution on fungal community dynamics is still poorly understood (Pointing and Hyde 2000). However, some studies carried out in contaminated salt marshes found that the fungal communities remain unchanged, which suggest a higher resistance and resilience of fungal and plant communities to these pollutants (Newell and Wall 1998; Newell et al. 2000b; Wall et al. 2001).

Conclusion

Despite diverse studies of salt marsh fungi, it is quite possible that the total fungal diversity associated with these ecosystems is not yet known. Neither are the abiotic and biotic factors

that determine the presence of a given fungal species. The continuing investment in this field of investigation is therefore fundamental, and should encompass different geographic regions and/or in other host plants in order to fill this knowledge gap.

1.3 Objectives

The overall purpose of this Ph.D. project is to contribute to filling knowledge gaps in diversity, biogeography and ecology of marine fungi, particularly of fungal communities associated with standing plants of one of the most dominant macrophytes of Portuguese temperate salt marshes, *Spartina maritima* (Curtis) Fernald. Specifically, this project intended to (1) assess the diversity of the fungal communities inhabiting *Spartina maritima* in two geographically and physically distinct salt marshes, (2) discriminate the ecological requirements of each fungal taxon (tolerance to fluctuating conditions of salinity and humidity, and nutritive preferences) and (3) infer about the potential ecological role of the most frequent fungi in the decomposition of the host plants.

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CHAPTER 2 – Diversity and ecological characterization of sporulating higher filamentous marine fungi associated with *Spartina maritima* (Curtis) Fernald in two Portuguese salt marshes

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Diversity and ecological characterization of sporulating higher filamentous marine fungi associated with *Spartina maritima* (Curtis) Fernald in two Portuguese salt marshes

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Abstract

Fungal communities associated with early stages of decomposition of *Spartina maritima* (Curtis) Fernald were assessed in two geographically distinct salt marshes in Portugal by direct observation of fungal sporulating structures. Twenty-three fungal taxa were identified from 390 plant samples, 11 of which were common to both study sites. *Natantispora retorquens*, *Byssothecium obiones*, *Phaeosphaeria spartinicola*, *Phoma* sp. 1 and *Stagonospora* sp. were the most frequent fungal taxa in the studied communities. The fungal species *Anthostomella spissitecta*, *Camarosporium roumeguerii*, *Coniothyrium obiones*, *Decorospora gaudefroyi*, *Halosarpheia trullifera*, *Leptosphaeria marina* and *Stagonospora haliclysta* were recorded for the first time on *S. maritima* plants; with the exception of *C. roumeguerii* and *L. marina*, all of these species were also new records for Portugal. The differences between species composition of the communities associated with *S. maritima* were attributed to differences in abiotic conditions of the salt marshes. Although the fungal taxa were distributed differently along the host plants, common species to both fungal communities were found on the same relative position, e.g. *B. obiones, Lulworthia* sp. and *N. retorquens* occurred on the basal plant portions, *Buergenerula spartinae, Dictyosporium pelagicum* and *Phoma* sp. 1 on the middle plant portions and *P. spartinicola* and *Stagonospora* sp. on the top plant portions. The distinct vertical distribution patterns reflected species-specific salinity requirements and flooding tolerance, but specially substrate preferences. The most frequent fungi in both communities also exhibited wider distribution ranges and produced a higher number of fruiting structures, suggesting a more active key role in the decay process of *S. maritima*.

Key words
Marine fungi; *Spartina maritima*; salt marsh; vertical distribution patterns; species-specific ecological requirements

Introduction

Marine fungi constitute an ecological group of fungi that colonize marine environments, ranging from intertidal zones to open ocean areas (Fell and Newell 1998). The greatest diversity of marine fungi, though, is found in estuarine ecotones, given the higher productivity and availability of substrates for colonization (e.g. Hyde and Lee 1995; Jones 2000; Sarma and Hyde 2001; Kohlmeyer et al. 2004; Kis-Papo 2005; Gessner et al. 2007; Alias et al. 2010). It is, in fact, in salt marshes and mangroves that saprobic marine fungi play a key role in the ecosystem´s ecological balance and dynamics by contributing to the degradation of complex organic matter and recycling of nutrients.

Spartina species, one of the most dominant primary producers in temperate salt marshes (Castillo et al. 2010), represent simultaneously one of the main substrates for saprobic marine fungi. These cordgrasses are highly enriched with lignocellulose (c.a. 75% of total biomass; Maccubbin and Hodson 1980; Hodson et al. 1984) and strictly depend on an active decomposition process to release the nutrients into the surrounding environment. This process is mainly triggered and carried out by ascomycetous fungi (e.g. Torzilli and Andrykovitch 1986; Bergbauer and Newell 1992; Newell 1996; Newell et al. 1996b; Newell and Porter 2000). Likewise in other grass-like plants, the major involvement of fungal species in the decomposition process occurs during the early stages when the senescent organs are still attached to standing-live plants, in natural positions (Newell and Fallon 1989; Newell et al. 1989; Samiaji and Barlocher 1996; Castro and Freitas 2000; Lyons et al. 2010). In fact, senescence and consequent decomposition processes may begin even before these plants have reached physiological maturity, occurring gradually from the outer and lower vegetative structures towards the inner and higher structures (Newell 2001a). Saprobic ascomycetous fungi were found to dominate the living microbial biomass on standing-decaying shoots of cordgrasses in the form of mycelia and reproductive structures, being the principal secondary producers (Newell et al. 1989, 1996a; Newell 1996, 2001a; Newell and Porter 2000).

The marine fungal colonizers of intertidal cordgrasses exhibit species-specific ecological patterns that determine their distribution on the plants. Barata (2002), Cornick et al. (2005), Kohlmeyer and Volkmann-Kohlmeyer (2001, 2002) and Kohlmeyer et al. (1995, 2005) considered the tidal regime, the vertical distribution of fungi in standing grasses and the definition proposed by Kohlmeyer and Kohlmeyer (1979) to distinguish between obligate and facultative marine fungi and set ecological boundaries; obligate marine fungi colonize preferentially lower portions of the plants, halotolerant terrestrial fungi inhabit aerial non-immersed parts and facultative marine fungi occur in between. The differentiation of these ecological groups of fungi based exclusively on this criterion is not easy or reliable in all circumstances since there are other factors interfering in the vertical distribution of fungi on host plants, such as plant tissue type (Sadaba et al. 1995; Barata 2002; Gessner et al. 2007) or interspecific competition (Newell and Porter 2000; Newell 2001a; Buchan et al. 2003). The relative subjectivity of the criterion to distinguish facultative from obligate fungi prompted Jones et al. (2009) to consider some of the described facultative fungal species as obligate fungal species. Nevertheless, on the dependence of personal opinion of the criterion, it is important to clarify the possible origin and ecological requirements of each fungus in order to better understand its role in functioning of the ecosystem.

According to the classification of Jones et al. (2009), the current list of obligate marine fungi associated with *Spartina* spp. includes 53 fungal species (Calado and Barata 2012); most of these species were identified on standing-decaying culms of *Spartina alterniflora* Loisel in North American salt marshes (e.g. Gessner 1977; Newell and Wasowski 1995; Samiaji and Barlocher 1996; Newell et al. 2000a; Newell 2001a; Buchan et al. 2002; Walker and Campbell 2010; Al-Nasrawi and Hughes 2012). A comparison among studies on different *Spartina* species in

geographically distant salt marshes using different morphological and/or molecular approaches highlighted a core group of marine fungi composed by the same ascomycetous species. Specifically, *Phaeosphaeria spartinicola*, *Mycosphaerella* sp. II and *Phaeosphaeria halima* have been mentioned as ubiquitous and dominant colonizers of *Spartina* leaf blades (e.g. Gessner 1977; Kolmeyer and Kohlmeyer 1979; Newell and Wasowski 1995; Newell and Zakel 2000; Newell et al. 2000a; Newell 2001a; Buchan et al. 2002, 2003; Lyons et al. 2010; Walker and Campbell 2010) and *Buergenerula spartinae* on leaf sheaths (Newell 2001a) and *Byssothecium obiones* on stems (Newell et al. 1996b; Barata 2002). These mentioned ascomycetous fungi were found to play an important functional role in the degradation of lignocellulosic secondary walls of plant cells (Benner et al. 1984; Torzilli and Andrykovitch 1986; Bergbauer and Newell 1992; Newell et al. 1996b; Newell and Porter 2000; Lyons et al. 2003). The presence of these fungal species on *Spartina* plants over a wide geographic range and the absence from other standing plants colonizing the same habitat suggested that these saprobic fungi are host-genus exclusive (Torzilli et al. 2006; Walker and Campbell 2010; Al-Nasrawi and Hughes 2012). Host exclusivity was proposed by Zhou and Hyde (2001) to apply in the cases of an exclusive occurrence of a strictly saprobic fungus on a particular or on a restricted range of related host plants, which does not reveal any symbiotic phase during its life cycle.

Although this core group of fungi is considerably well-known, in terms of species composition and general ecological preferences, there are still gaps in understanding the ecology of each fungal species and its specific role on decomposition process.

South European salt marshes are dominated by *Spartina maritima* (Curtis) Fernald, one of the main primary producers of these ecosystems (Castillo et al. 2008; Sousa et al. 2008), and the marine mycota associated with this plant has been surprisingly poorly investigated. Barata (2002) surveyed *S. maritima* standing plants from three salt marshes situated in the central west coast of Portugal and identified 20 fungal taxa; in one of these salt marshes, Barata (2006) recorded 26 colonizers of *S. maritima* baits exposed to different submersion conditions. Azevedo et al. (2012) also inventoried the saprobic marine mycota associated with *S. maritima*, but from drift substrates collected in four Portuguese west coast beaches; 31 fungal taxa were recorded on *S. maritima* stems. Although the fungal community associated with standing plants and drift stems included some common fungal species and belonging to the core group, both substrates were dominated by different fungal species (Barata 2002; Azevedo et al. 2012).

Therefore, and in a general perspective, the present study intends to be the first comprehensive study of fungi associated with *S. maritima* in Portugal, providing key information on ecological requirements of fungi inhabiting standing-live plants.

Specifically, this study aims to contribute to: (1) the inventory of higher filamentous marine fungi associated with S. maritima, (2) a better understanding of ecology and functional role of fungi in early stages of decomposition of *S. maritima* and (3) the evaluation of the effects of seasonality and environmental parameters on fungal community by comparing two Portuguese salt marshes with distinct geographical locations, biophysical structures, anthropogenic pressures and representativeness of this host plant. Fungal species were identified by direct observation

of the reproductive structures (traditional microscopy-based methods) and then classified into obligate or facultative marine fungi based on the average vertical position on plants and salt requirements for growth assessed by a culture-dependent assay.

Material and methods

Study sites

The study was conducted in two salt marshes: the Guadiana estuary (Castro Marim) situated in the southeastern coast (37.23° N, 7.42° W) in the Mediterranean region (Costa et al. 2009) and the Ria de Aveiro coastal lagoon located in the northwest of Portugal (40.62° N, 8.74° W) included in Eurosiberian region (Costa et al. 2009) (Fig. 1).

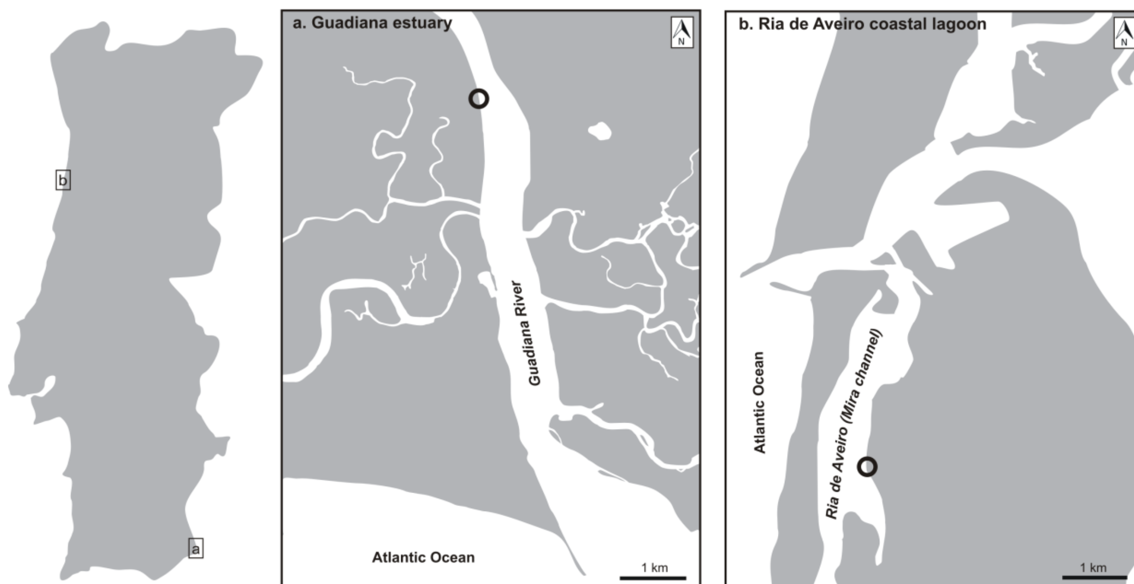


Fig. 1 Study sites: Guadiana estuary (a) and Ria de Aveiro coastal lagoon (b); *black circle* markers indicate collection areas in the salt marshes

Both ecosystems are mesotidal, with a mean tidal range of 2.0 m, and have predominantly semi-diurnal tides that dominate the hydrodynamics of the systems (Morales 1997; Dias et al. 1999). However, the two study sites exhibit a different physical configuration. Lower Guadiana estuary consists of a narrow channel bordered by marsh ecosystems, which is oriented perpendicular to the coast and connects the fluvial channel with the open littoral zone (Morales 1997). Ria de Aveiro coastal lagoon runs parallel to the coastline and consists of a complex network of channels surrounded by mud flats and salt marshes; the lagoon is permanently connected to the Atlantic Ocean by a deep and narrow artificial channel (Dias et al. 1999, 2000). The freshwater flow in both systems is also different; Guadiana estuary receives a high

input of freshwater from Guadiana river whereas Mira channel in Ria de Aveiro lagoon receives a lower freshwater discharge from a small system of ponds and rivers (Dias and Lopes 2006; Kilsby et al. 2007).

Additional details of study sites are summarized in Table 1.

Table 1 Abiotic conditions in Guadiana estuary and Ria de Aveiro coastal lagoon

	Guadiana estuary	Ria de Aveiro coastal lagoon
Minimum tidal range (neap tides) [a]	1.2 m	0.6 m
Maximum tidal range (spring tides) [a]	2.8 m	3.2 m
Tidal currents velocities [a]	0.5 ms^{-1}	1.0 ms^{-1}
Mean sea surface temperature [b]	18.5 °C	16.5 °C
Salinity [c]	0–33 ppt [d]	25–35 ppt [e]
pH [c]	7.5–8.2	7.7–8.6

[a] Parameters described in Morales (1997) and Dias et al. (1999)

[b] Parameter obtained in National Oceanic and Atmospheric Administration (NOAA)

[c] Parameters measured at the collection areas during the sampling periods, including measurements made in January and May 2001 by Caetano et al. (2006) [d], in July 1996 by Dias and Lopes (2006) [e] and in March 2009 by Rodrigues et al. (2012) [e] near the collection areas

The conservation state of both study sites is also different as a result of different conservation status and anthropogenic pressure. Guadiana estuary is protected as a Natural Reserve, being subjected to less negative human impacts (Caetano et al. 2006, 2007). In contrast, Ria de Aveiro lagoon was for decades (and until 1994) the main receptor of highly contaminated effluent discharges (Pereira et al. 2009; Oliveira et al. 2010), and a relatively low mercury fraction is still present in the water column, sediment and biota (Coelho et al. 2009, 2014; Pereira et al. 2009).

Host plant

In addition to being one of the main primary producers, *S. maritima* represents an important pioneer grass that occupies the first level of emerged vascular vegetation. Given its rhizomatous nature, it assumes a fundamental role in the protection of coastline from erosion by trapping and aggregating sediment within the clumps (Castellanos et al. 1998; Sánchez et al. 2001; Ferreira de Carvalho et al. 2013) and in the reduction of eutrophication of the system by sequestering nutrients and metals from sediments (Caetano et al. 2007; Sousa et al. 2008; Curado et al. 2013, 2014). *S. maritima* communities include distinguishable tall and short growth forms, which have been attributed to genotypic differences (Sánchez et al. 1997; Otero et al. 2000) and phenotypic plasticity to different environmental conditions (Castillo et al. 2005). In Castro Marim salt marsh, *S. maritima* plants are shorter (average plant height 39 ± 5 cm) and with more inrolled and smaller leaf blades (1/3 of total plant height) whereas in Ria de Aveiro salt marsh, plants are taller (average plant height 49 ± 6 cm) and with more expanded and

larger leaf blades (1/2 of total plant height). In addition to intraspecific differences in morphology, both communities exhibited different distribution patterns; in Castro Marim salt marsh, *S. maritima* community forms extensive monotypic beds along the riverside whereas in Ria de Aveiro salt marsh, community is fragmented and disrupted in relatively small and dispersed patches.
Although both communities of *S. maritima* follow the natural phenological cycle, with a growing season occurring during spring to early summer, plants of different maturation phases are present throughout all seasons.

Sampling procedure

Mature standing-live plants experiencing the same daily tidal wet-dry cycles (i.e. occupying the same topographic level, with similar height and containing green, senescent and early-decay plant tissues) were randomly collected in Castro Marim and Ria de Aveiro salt marshes (2.45 and 2.37 m above the Portuguese hydrographic zero, respectively), bimonthly over a 2-year period (October 2010 to August 2012). Twenty plants were collected each of the first 3 sampling periods and 15 plants afterwards (a total of 390 plants). Five additional plants were also collected in each period of the last sampling year (February 2012 to August 2012) for isolation of marine fungi (a total of 20 plants). Plant samples were placed in plastic bags and returned to laboratory.

Morphology-based species identification

The collected plants were carefully rinsed with running tap water to remove fine-grained sediments and seaweeds and air-dried. Each air-dried plant was sequentially analysed from the basal to the top portion and from the external vegetative structures (leaf sheaths and blades) towards the more internal structures (stems). Fungal structures (fruit bodies, spores and hyphopodia) observed on each vegetative structure were picked up under a dissecting microscope (Wild M8) mounted into a drop of sterile seawater on a slide examined under a light microscope (Leitz Laborlux S, with Normaski) with detailed morphology recorded. The fungi were identified using the dichotomous keys of Kohlmeyer and Kohlmeyer (1979), Kohlmeyer and Volkmann-Kohlmeyer (1991), Hyde and Sarma (2000) and Jones et al. (2009). The vertical position of identified fungi was also recorded, as well as the density of produced fungal structures; for more than 10 fungal structures per square centimeter of colonized vegetative structure, the density was considered high. The fungal structures were photographed and preserved on microscope slides after replacement of seawater by glycerin and sealed with several layers of nail varnish. Moreover, some of the identified fungal structures were maintained on the original dry plant material and included in the personal collection of M. Barata (Herbarium of the University of Lisbon - LISU).

Isolation of marine fungi and preservation of pure cultures

Cultures were obtained by single spore method, according to the conventional procedures of Vrijmoed (2000). Five fruiting structures (ascomata or pycnidia) of a given fungal taxon growing on fresh plant materials were transferred into a drop of sterile seawater on a microscope slide and squashed to force the discharge of the spores. This suspension of spores was then transferred with a Pasteur pipette onto gridded plates containing cornmeal agar made with aged diluted seawater (CMA/sw 50%) and supplemented with chloramphenicol (0.05%), one drop per square. Plates were incubated at room temperature for 1–2 days until germination of the spores. Each germinated spore was then transferred onto a new CMA/sw plate.
In order to establish a culture collection, each isolated fungus was subcultured and preserved by three methods: (1) one colony was maintained on CMA/sw plate at 4 °C, (2) plugs removed from the growing margin of four colonies were transferred to McCartney bottles filled with sterile diluted seawater (50%) and kept at 4 °C, and (3) to cryotubes filled with glycerol (10%) and stored at -80 ºC.

Growth rates

Growth rates of selected fungi were determined in cornmeal agar media made with diluted seawater (CMA/sw 50%) and with distilled water (CMA/dw) at room temperature (18–25 °C). With this purpose, an agar disc was cut from the growing edge of fungal colonies and inoculated at the intersection point of two perpendicular lines previously drawn on the bottom of CMA/sw 50% and CMA/dw plates; three replicates were performed for each monospore isolate. The colony growth was assessed every 2 days, for 30 days, by measuring and averaging the colony diameter along the two perpendicular axes.

Data analyses

Diversity indices

The following diversity indices were calculated in order to better characterize and compare the fungal communities inhabiting Castro Marim and Ria de Aveiro salt marshes: Shannon diversity index ($H'=-\sum_{i=1}^{S} pi\ln(pi)$, where *s* is the number of fungal taxa in the community and *pi* is the proportion of occurrences of fungal taxon *i* relative to total number of occurrences), Shannon´s equitability index ($E=H'/Hmax$, where $H_{max}=\ln s$) and Sorenson similarity index ($SI=2j/(a+b)$, where “j” is the number of common fungal taxa to both sites, *a* is the number of fungal taxa in one site and *b* is the number of fungal taxa in the other site). The comparison of Shannon diversity indices between study sites was performed based on randomization procedures of bootstrapping using the PAST v2.17c statistical software (Hammer et al. 2001). P values were

estimated by resampling and randomly redistributing the data 1000 times (Efron and Tibshirani 1986); differences were considered statistically significant for p value<0.05.
The average number of fungal taxa per plant sample (total number of fungal occurrences divided by the total number of plant samples) was also determined for Castro Marim and Ria de Aveiro salt marshes and for the assembly of the two salt marshes.

Frequencies of occurrence and vertical distribution patterns – Total and in each sampling period

The percent frequency of occurrence for each taxon in the fungal community was assessed (number of plant samples colonized by a specific fungus divided by the total number of plant samples x 100). Fungal taxa were grouped according to the percent frequency of occurrence and the classification proposed by Tan et al. (1989) in very frequent (>20%), frequent (10–20%) and infrequent (<10%).
The average vertical distribution data of common fungal taxa in both study sites were compared by Student´s *t* tests, using IBM SPSS v22.0 statistical software (IBM Corporation, Somers, NY).
In an attempt to better discriminate vertical distribution patterns and ecological requirements of fungal taxa in the two salt marshes, three vertical microhabitats were defined by separating the plant samples in three equally portions based on maximum plants height (basal 0–20 cm, middle >20–40 cm, top >40–60 cm). For each plant portion, the same diversity indices (Shannon, Equitability and Sorensen similarity indices) were determined; comparisons among Shannon diversity indices in plant portions were performed adopting the same procedures described above. Additionally, frequencies of occurrence of each fungal taxon, in each sampling period, in each plant portion were calculated. These dataset matrices were used to perform a preliminary Cluster Analysis with Bray-Curtis similarity measure; seven samples were considered outliers, given the atypical and divergent behavior and excluded from the subsequent analyses. The reconstructed dataset matrices were then used to perform another cluster analysis and a detrended correspondence analysis, using the PAST v2.17c statistical software.
The effect of seasonality on fungal communities was assessed by analysing the variations of the frequencies of occurrence and vertical positions of all fungal taxa during the two sampling years; the former parameters were interpreted graphically, and the second parameters were tested statistically using IBM SPSS v22.0 software. A two-way analysis of variance (ANOVA) was performed in order to test the effect of sampling periods and fungal taxa on the total variations of vertical distributions in both communities. After this procedure, a new one-way ANOVA was performed for each taxon to evaluate the statistical significance of its vertical distribution variation.

Flooding regime in Castro Marim and Ria de Aveiro study sites

The flooding conditions in both study sites were assessed, given the differences in the physical configuration of intertidal systems and in the morphology of host plant. The percentage of days in each month that *Spartina* plants were totally submerged, at least once, was determined, considering average plants height, tidal range (high tides height) and the topographic position of the plants on both salt marshes. The average time per day that the plants remained flooded at each sampling site was estimated using a model developed by Serôdio and Catarino (2000). The frequency and time length of flooding were also determined for basal, middle and top plant portions.

Vegetative growth rates of fungal isolates

The growth rates were extracted from linear regression equation of colony diameter increase over 30 days. The differences between growth rates in the two culture media were assessed with Student´s *t* tests, using IBM SPSS v22.0 statistical software.

Results

Fungal diversity

Twenty-three sporulating higher filamentous marine fungi were recorded from the total 390 analysed plants, with 20 and 14 fungal taxa occurring in Castro Marim and Ria de Aveiro salt marshes, respectively (Table 2; Fig. S1, Online Resource). The average number of fungi per plant was found to be five in both sites.

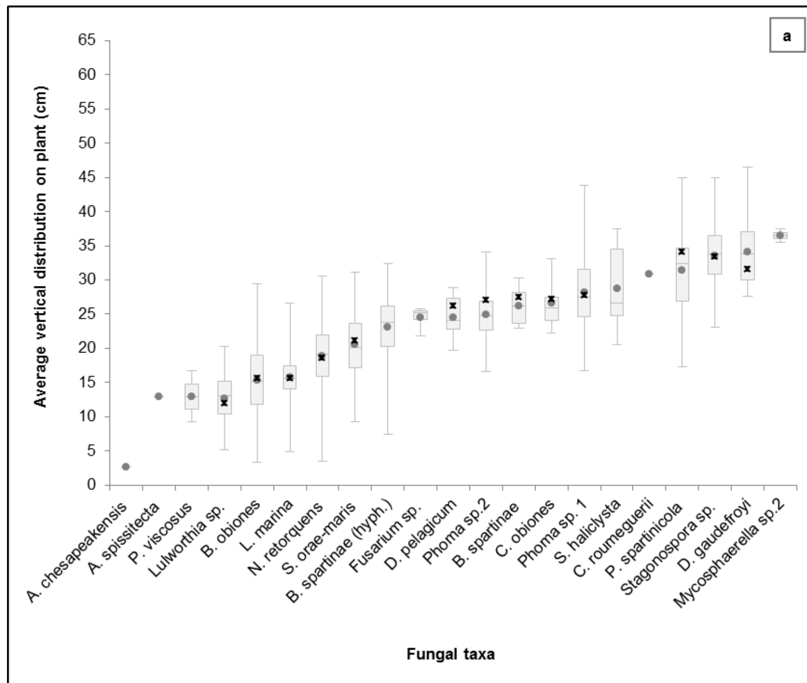
Table 2 Percent frequency of occurrence of fungal taxa and species diversity indices in communities in Castro Marim and Ria de Aveiro salt marshes and on average between the sites; fungal taxa are organized by decreasing values of frequency of occurrence based on the average and according to the three categories proposed by Tan et al. (1989)

Fungal taxa	Percent frequency of occurrence		
	Overall (390 plants)	Castro Marim (195 plants)	Ria de Aveiro (195 plants)
Very frequent (>20 %)			
Natantispora retorquens (Shearer & J.L. Crane) J. Campb., J.L. Anderson & Shearer	95.1	91.8	98.5
Phaeosphaeria spartinicola Leuchtm.	87.4	83.1	91.8
Byssothecium obiones (P. Crouan & H. Crouan) M.E. Barr	74.1	86.7	61.5
Phoma sp. 1	50.5	56.4	44.6
Stagonospora sp.	39.7	38.5	41.0
Mycosphaerella sp. I	38.7	0.0	77.4
Lulworthia sp.	27.2	9.7	44.6
Buergenerula spartinae Kohlm. & R.V. Gessner	25.9	37.4	14.4
Frequent (10–20 %)			
Sphaerulina orae-maris Linder	20.0	39.5	0.5
Leptosphaeria marina Ellis & Everh.	15.4	30.8	0.0
Infrequent (<10 %)			
Decorospora gaudefroyi (Pat.) Inderb., Kohlm. & Volkm.-Kohlm.	6.2	12.3	0.0
Phoma sp. 2	5.9	11.8	0.0
Coniothyrium obiones Jaap	2.6	5.1	0.0
Dictyosporium pelagicum (Linder) G.C. Hughes ex E.B.G. Jones	2.6	3.1	2.1
Stagonospora haliclysta Kohlm.	2.1	4.1	0.0
Fusarium sp.	1.0	2.1	0.0
Aniptodera chesapeakensis Shearer & M.A. Mill.	0.8	0.5	1.0
Panorbis viscosus (I. Schmidt) J. Campb., J.L. Anderson & Shearer	0.5	1.0	0.0
Anthostomella spissitecta Kohlm. & Volkm.-Kohlm.	0.3	0.5	0.0
Camarosporium roumeguerii Sacc.	0.5	0.5	0.5
Halosarpheia trullifera (Kohlm.) E.B.G. Jones, S.T. Moss & Cuomo	0.3	0.0	0.5
Leptosphaeria sp.	0.3	0.0	0.5
Mycosphaerella sp. 2	0.5	1.0	0.0
Species richness (S´)	23	20	14
Number of occurrences	1940	1006	934
Average number of fungi per plant	4.97	5.16	4.79
Shannon diversity index (H´)	2.33	2.32	2.02
Equitability index (E)	0.74	0.78	0.77

The fungal communities of Castro Marim and Ria de Aveiro salt marshes were mostly composed of the Ascomycota, representing 60% (12) and 71% (10) of the total number of fungal taxa and 76% (769) and 82% (762) of the number of occurrences, respectively. The remaining fungal taxa found in both study sites belong to asexual fungi, mainly coelomycetes (30% in Castro Marim and 21% in Ria de Aveiro). Ascomycetous fungi were restricted to Dothideomycetes, Sordariomycetes and Sordariomycetes *incertae sedis*; Pleosporales, Microascales (i.e. Halosphaeriaceae) and Capnodiales were the most representative orders, with 33, 25 and 17% in Castro Marim and 20, 30 and 20% in Ria de Aveiro, respectively.
Although the diversity of fungal community was significantly higher in Castro Marim than Ria de Aveiro ($p<0.01$), both communities revealed a similar high equitability value.
The results also evidenced a high overlap between fungal communities of Castro Marim and Ria de Aveiro salt marshes regarding species composition and common taxa. From the 23 total fungal taxa associated with *S. maritima*, 48% (11) were common between the study sites. *Natantispora retorquens*, *B. obiones*, *P. spartinicola*, *Phoma* sp. 1 and *Stagonospora* sp. were very frequent in both communities. Sorensen´s index revealed a similarity of 0.65 between both fungal communities.
The main differences between the two fungal communities were the number of exclusive infrequent fungal taxa, which was higher in Castro Marim than in Ria de Aveiro salt marsh (8 vs 2). Moreover, the former study site included two frequent and one very frequent exclusive fungal taxa, namely *Leptosphaeria marina*, *Decorospora gaudefroyi* and *Phoma* sp. 2, respectively, while Ria de Aveiro harboured only one very frequent exclusive fungal species, *Mycosphaerella* sp. I. *Sphaerulina orae-maris* was very frequent in Castro Marim and infrequent in Ria de Aveiro salt marsh.

Vertical distribution of fungi

Fungal taxa inhabiting *S. maritima* in both study sites were distributed vertically along the plant, displaying distinct distribution patterns; some were restricted to the upper or lower portions of the plants while others spanned widely along the plant, showing different extents of substrate occupation (Fig. 2).



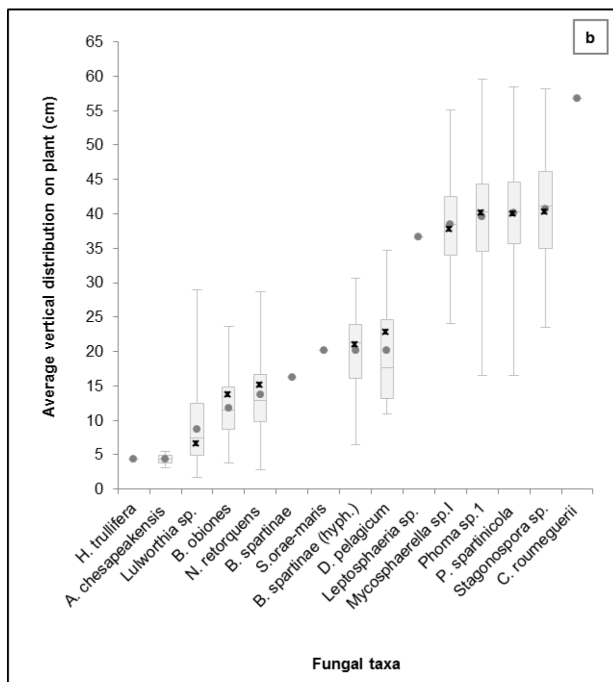


Fig. 2 Vertical distribution of fungal taxa on standing *S. maritima* in Castro Marim (a) and Ria de Aveiro (b) salt marshes. The *boxplot* shows the distribution of the average vertical positions of each fungal taxon on all plant samples: the quartiles include 50% of the distribution, and the *whiskers* indicate the spread of the data outside the upper and lower quartiles. The *grey circle* (●) represents the average of the average vertical positions on all samples. The *black marker* (×) represents the average vertical position where the density of fruiting structures is higher in all plant samples (for the majority of the rare or infrequent fungal taxa, it was not observed a high number of fruiting structures on the plant samples, and for this reason this information is lacking in the figure). *B. spartinae* differentiated hyphopodia and ascomata at different vertical levels on the plant, being divided in two groups accordingly with the type of structure

In general, the fungi produced a higher number of reproductive or other fungal structures at the average vertical position of their distribution (Fig. 2).

A comparison between vertical distribution data of common fungal taxa in Castro Marim and Ria de Aveiro salt marshes demonstrated that the differences were statistically significant (p<0.05), except for *Dictyosporium pelagicum*. However, the shared fungal taxa appeared to occupy the same relative vertical position on the plants, despite the variations on the absolute vertical distribution.

Fungal subcommunities on basal, middle and top portions of the plants in both study sites were shown to be considerably different by diversity indices (Table 3), cluster analysis (Fig. 3) and Detrended Correspondence Analysis (DCA; Fig. 4).

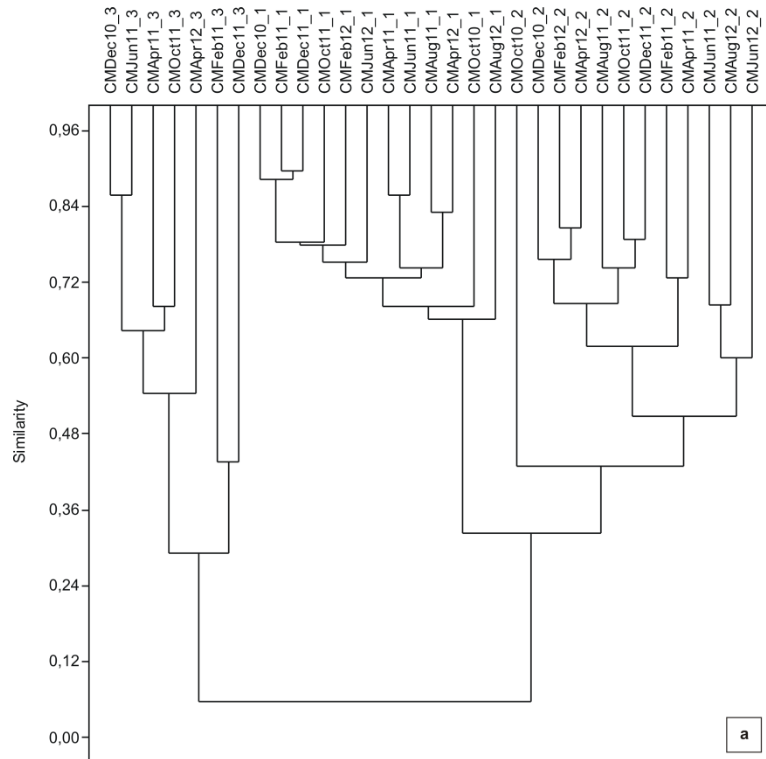
Table 3 Diversity indices and number of records in the 3 vertical portions of the plants in Castro Marim and Ria de Aveiro salt marshes

	Castro Marim			Ria de Aveiro		
	Basal	**Middle**	**Top**	**Basal**	**Middle**	**Top**
Species richness (S´)	13	18	4	10	11	5
Number of records	380	542	20	388	301	237
Shannon index (H´)	1.68	2.35	1.30	1.28	1.78	1.35
Equitability index (E)	0.66	0.81	0.94	0.56	0.74	0.84
	Basal x Middle	**Middle x Top**	**Top x Basal**	**Basal x Middle**	**Middle x Top**	**Top x Basal**
Sorensen similarity index	0.65	0.36	0.24	0.67	0.50	0.27

Middle portion yielded the highest species richness and diversity than either basal or top portions (Table 3). The differences in the Shannon indices were statistically significant between basal/middle and middle/top portions (p<0.001), but not between basal/top portions in Castro Marim and Ria de Aveiro salt marshes (p>0.05).

The fungal subcommunities inhabiting basal and middle plant portions revealed higher similarities considering species richness and fungal taxa composition in both study sites than those in basal/top portions and in middle/top portions.

The cluster and DCA analyses, which provided an integrated overview of spatial arrangement of fungal community based on the frequencies of occurrence of fungal taxa in each vertical plant portion corroborated the existence of three distinct microhabitats supporting distinct fungal subcommunities (Figs. 3 and 4).



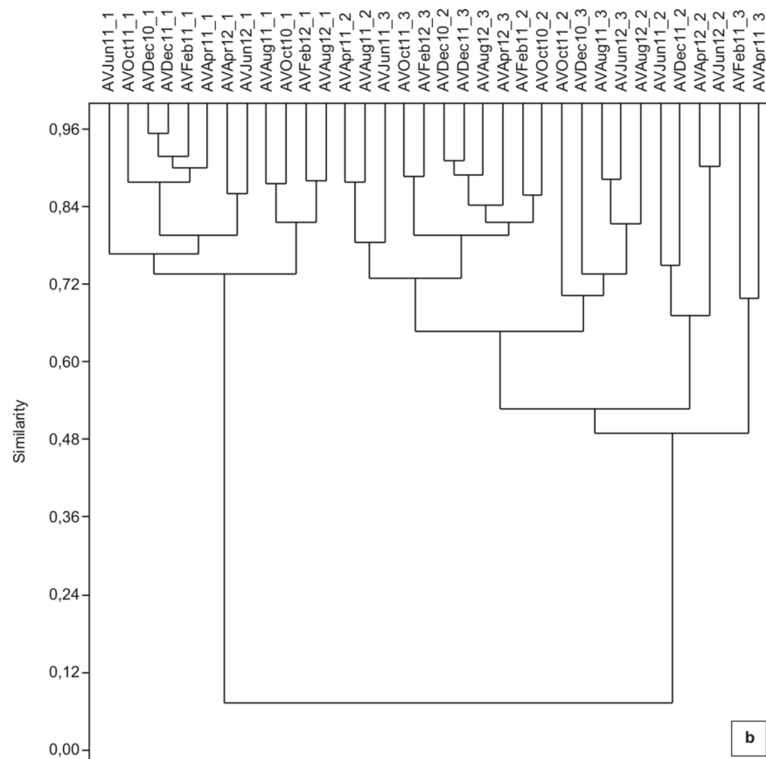


Fig. 3 Cluster dendrogram based on Bray-Curtis similarity of fungal communities colonizing basal, middle and top plant portions in each sampling period and study site (**a** Castro Marim, **b** Ria de Aveiro), considering the frequency of occurrence of fungal taxa. The *first two letters* of the code name indicate the study site (*CM* Castro Marim, *AV* Ria de Aveiro), the *next three letters* and *two numbers* designate the month and year of the collection respectively, and the *last number* indicates the plant portion (*1* basal, *2* middle, *3* top)

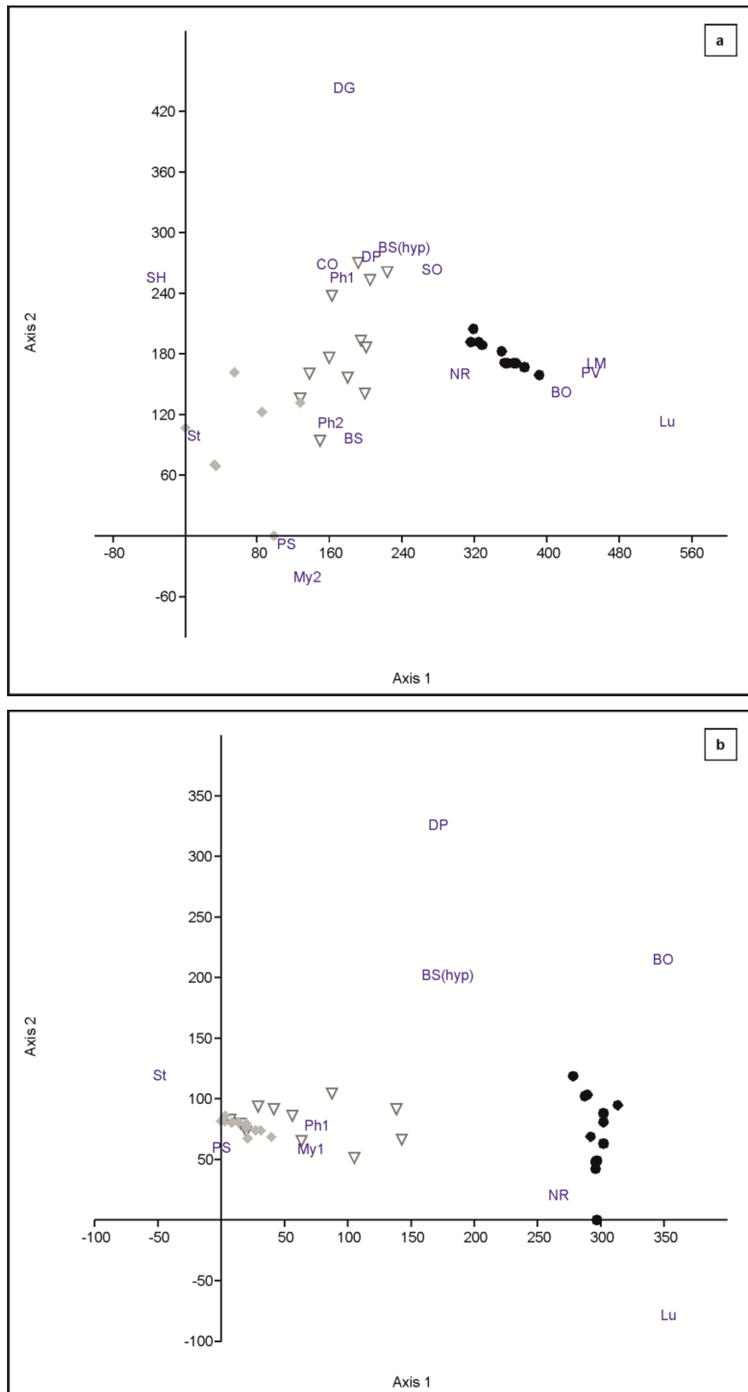


Fig. 4 Two-dimensional DCA plot expressing the fungal taxa and the three vertical plant portions spatial distributions based on frequency of occurrence of fungal taxa in each portion in each sampling period and in each study site (**a** Castro Marim, **b** Ria de Aveiro). The *black pentagons* (⬟), *dark grey triangles* (▽) and *light grey polygons* (◆) correspond to basal, middle and top plant portions, respectively. The *two-letter code* represent fungal taxa: *AC Aniptodera chesapeakensis*, *BO Byssothecium obiones*, *BS Buergenerula spartinae* ascomata, *BS(hyp) Buergenerula spartinae* hyphopodia, *CO Coniothyrium obiones*, *CR Camarosporium roumeguerii*, *DG Decorospora gaudefroyi*, *DP Dictyosporium pelagicum*, *LM Leptosphaeria marina*, *Lu Lulworthia* sp., *My1 Mycosphaerella* sp. I, *My2 Mycosphaerella* sp. 2, *NR Natantispora retorquens*, *Ph1 Phoma* sp. 1, *Ph2 Phoma* sp. 2, *PS Phaeosphaeria spartinicola*, *PV Panorbis viscosus*, *SH Stagonospora haliclysta*, *SO Sphaerulina orae-maris*, *St Stagonospora* sp.

The cluster analysis (Fig. 3) performed with Castro Marim dataset separated first the top plant portion (ca. 0.29 of similarity) and then basal (ca. 0.31 of similarity) from middle portion, coinciding exactly with the defined microhabitats; with Ria de Aveiro dataset, the analysis only distinguished clearly the basal plant portion from the middle and top portions (ca. 0.42 of similarity).

The DCA reinforced the results from the previous analysis (Fig. 4). Along the axis 1, with the higher eigenvalue (Castro Marim: 0.52; Ria de Aveiro: 0.86) and explanatory power, there was a clear spatial separation of the three plant portions in Castro Marim dataset, which was not so evident between middle and top portions in Ria de Aveiro dataset. The graphical separation of basal, middle and top portions followed the natural vertical sequence of microhabitats, which confirmed the higher similarity of fungal subcommunities between adjacent plant portions.

The DCA analysis also highlighted specific ecological niches by plotting the distribution of fungal taxa across plant portions. Fungal taxa were distributed along the axis 1 following the vertical distribution on the standing plants in Castro Marim and Ria de Aveiro salt marshes, from the top to the basal plant portions. The spatial proximity of each fungal taxon to a certain plant portion in the plot suggested higher affinities to that particular microhabitat. Thus, the results evidenced a subcommunity associated with basal portions, mainly represented by *B. obiones, Lulworthia* sp. and *N. retorquens* in both salt marshes, and *Panorbis viscosus* and *L. marina* in Castro Marim; a subcommunity colonizing middle portions composed by *B. spartinae, D. pelagicum* and *Phoma* sp. 1 in both study sites and *Coniothyrium obiones*, *D. gaudefroyi, Phoma* sp. 2 and *S. orae-maris* in Castro Marim; and a subcommunity associated with upper portions composed by *P. spartinicola* and *Stagonospora* sp. in both salt marshes, *Mycosphaerella* sp. I in Ria de Aveiro, and *Mycosphaerella* sp. 2 and *Stagonospora haliclysta* in Castro Marim.

Both axes 2 and 3 presented a lower eigenvalue in Castro Marim (axis 2: 0.12; axis 3: 0.06) and Ria de Aveiro (axis 2: 0.08; axis 3: 0.06) datasets, explaining little variation in the data.

Comparisons of micro-environmental conditions on the three plant portions revealed some differences. Specifically, it was observed a decrease in flooding time (from 8 to 2 daily hours) and frequency (from 100 to 50% of the days per month) along the vertical axis of the plants, from the basal upwards to the top portions, in both salt marshes; although it was not measured in this study, this vertical gradient of tidal flooding reflected obviously in salinity and water availability levels in each plant portion. Middle and top plant portions in Castro Marim salt marsh remained slightly longer and were more frequently submerged than analogous portions in Ria de Aveiro salt marsh. Furthermore, it was found that the vegetative structures in each plant portion were different: basal portions included mostly a senescent naked stem or a stem enwrapped by leaf sheaths; middle portions included mainly a stem enwrapped by leaf sheaths and leaf blades; top portions included mostly upright-standing leaf blades. Nevertheless, the host plants from the two salt marshes presented some differences in the proportions of vegetative structures included in analogous portions, as a result of differences in the morphology. The main differences were found in the middle plant portions; Castro Marim plants

included mostly the stem and leaf sheaths whereas Ria de Aveiro plants also included leaf blades in this portion.

Seasonality

The effects of seasonality on fungal community dynamics and particularly in the frequencies of occurrence of the most frequent fungal taxa in the two study sites (Fig. 5) and for fungi producing a high density of fruiting structures (Fig. 6) were investigated.

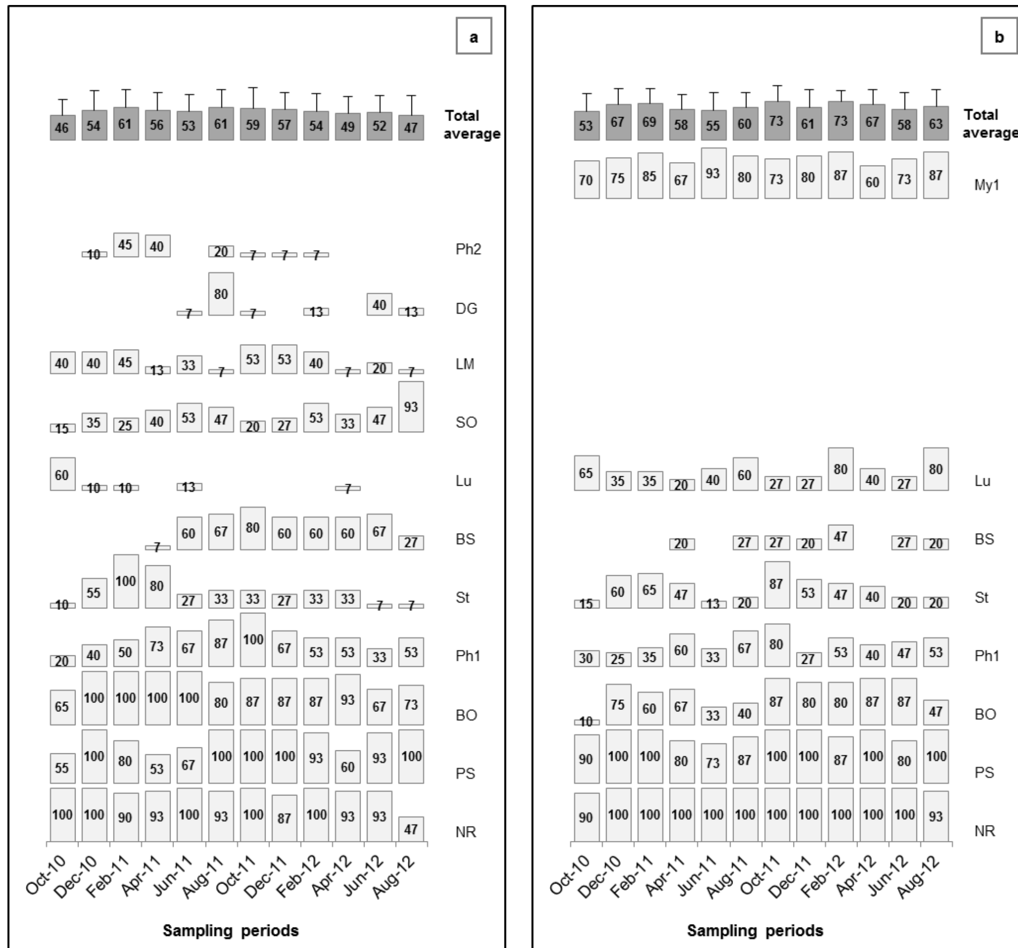


Fig. 5 Bimonthly variation of the frequency of occurrence (%) of each fungal taxon in Castro Marim (a) and Ria de Aveiro (b) salt marshes and average variation of the frequencies of occurrence in each sampling period: *BO Byssothecium obiones*, *BS Buergenerula spartinae*, *DG Decorospora gaudefroyi*, *LM Leptosphaeria marina*, *Lu Lulworthia* sp., *MyI Mycosphaerella* sp. I, *NR Natantispora retorquens*, *Ph1 Phoma* sp. 1, *Ph2 Phoma* sp. 2, *PS Phaeosphaeria spartinicola*, *SO Sphaerulina orae-maris*, *St Stagonospora* sp.

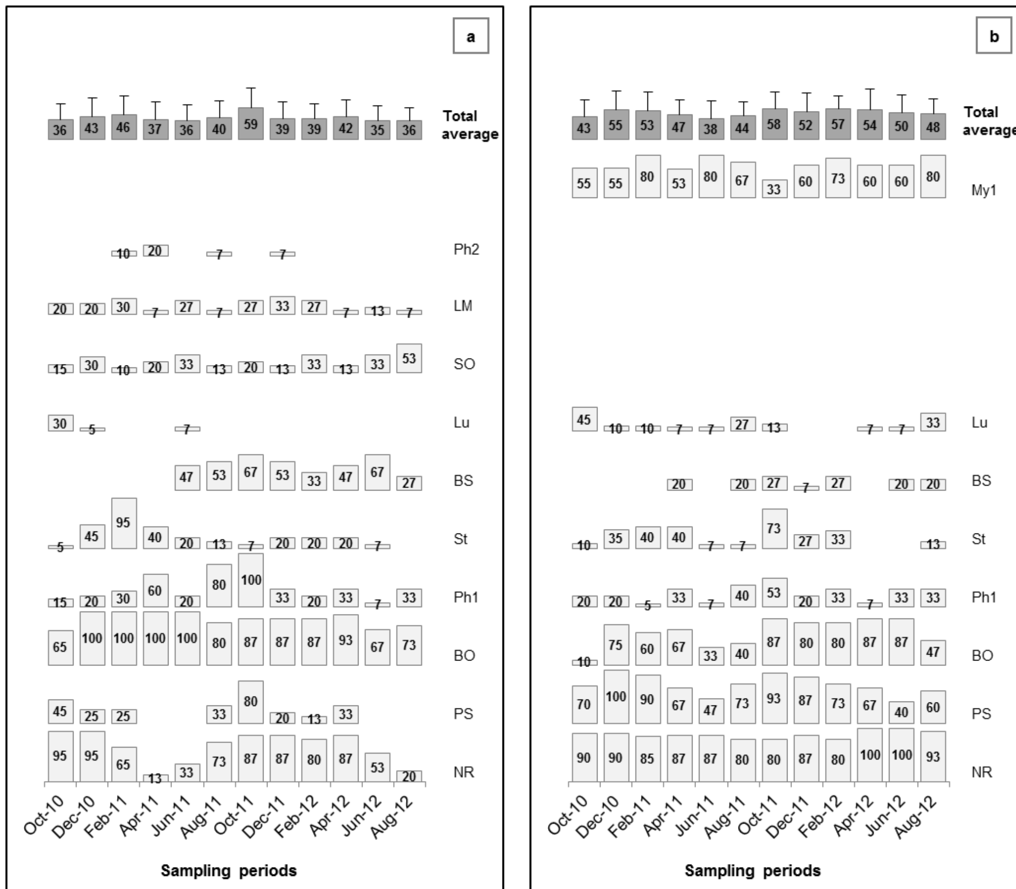


Fig. 6 Bimonthly variation of frequency of occurrence (%) of high density of fungal structures produced by each fungal taxon in Castro Marim (a) and Ria de Aveiro (b) salt marshes and average variation of the frequencies of occurrence in each sampling period: *BO Byssothecium obiones*, *BS Buergenerula spartinae*, *LM Leptosphaeria marina*, *Lu Lulworthia* sp., *My1 Mycosphaerella* sp. I, *NR Natantispora retorquens*, *Ph1 Phoma* sp. 1, *Ph2 Phoma* sp. 2, *PS Phaeosphaeria spartinicola*, *SO Sphaerulina orae-maris*, *St Stagonospora* sp. *Buergenerula spartinae* only differentiated a high density of hyphopodia and not fruiting structures

The results showed that seasonally driven changes in environmental conditions apparently had no significant effect on the presence and life cycle of *N. retorquens* in Ria de Aveiro salt marsh, but interfered slightly on the presence and production of fruiting structures of the remaining fungi for both communities (Figs. 5 and 6).

Although no obvious species-specific seasonal patterns were detected, the presence of *P. spartinicola* and *Stagonospora* sp. on *Spartina* plants in Castro Marim and Ria de Aveiro salt marshes was generally lower during warmer months than in cooler periods. Similarly, for these mentioned fungal taxa and also for *N. retorquens* in Castro Marim salt marsh, it was observed a decrease in the production of fruiting structures during the spring–summer seasons.

Despite the seasonal effect on fungal communities, the dominance pattern was maintained in both salt marshes, i.e. the most frequent fungi were the same over time. In addition of being omnipresent in the communities, these fungi were also investing more intensively in sexual or asexual reproduction and/or differentiating other fungal structures in Castro Marim and Ria de Aveiro salt marshes (Figs. 5 and 6).

Seasonal variation on the vertical positions of fungal taxa that occurred during the sampling periods on the plants was also investigated (Fig. 7).

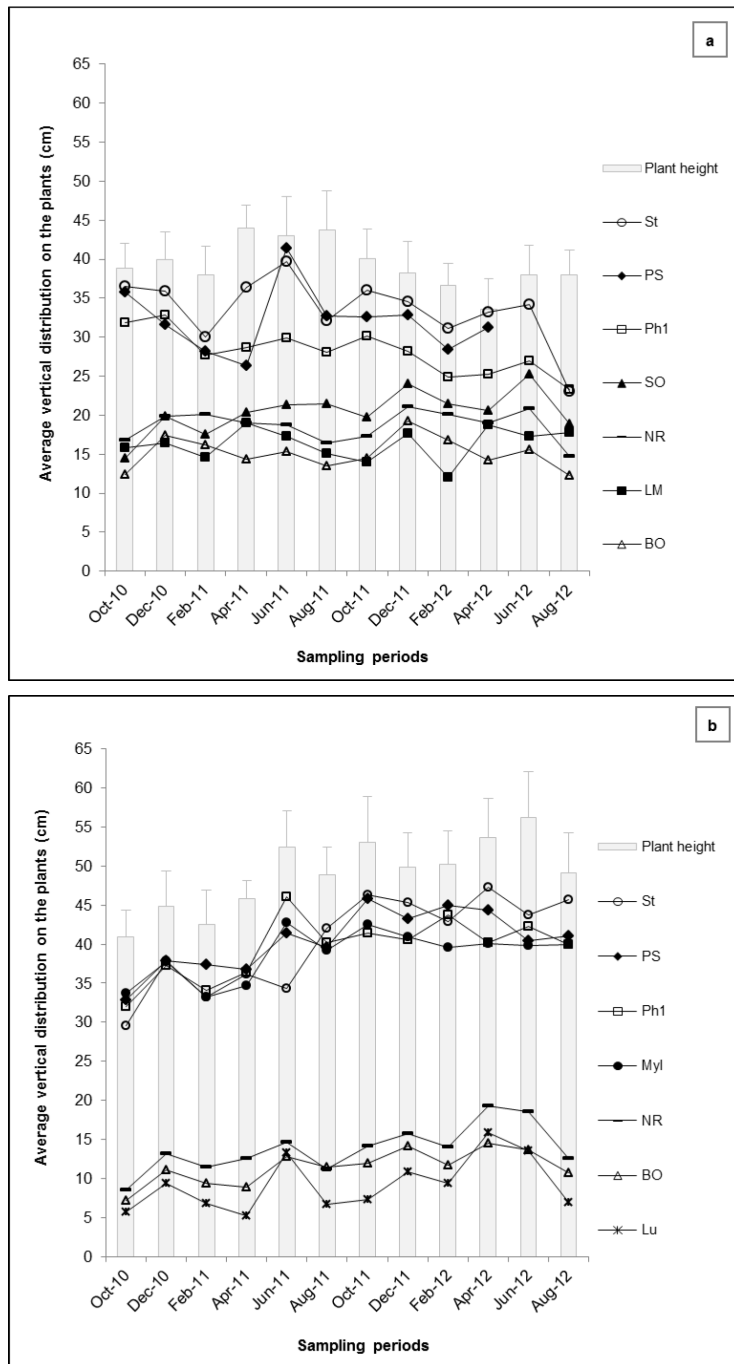


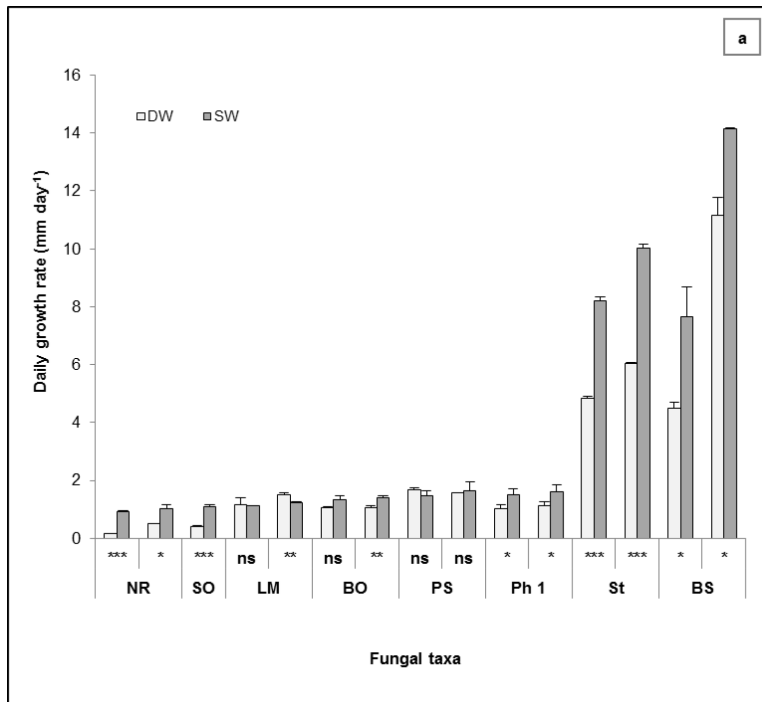
Fig. 7 Bimonthly variation in the average height of host plants and vertical position of fungal taxa that occurred in most of the sampling periods on the plants in Castro Marim (a) and Ria de Aveiro (b) salt marshes: *BO Byssothecium obiones*, *LM Leptosphaeria marina*, *Lu Lulworthia* sp., *My1 Mycosphaerella* sp. I, *NR Natantispora retorquens*, *Ph1 Phoma* sp. 1, *PS Phaeosphaeria spartinicola*, *SO Sphaerulina orae-maris*, *St Stagonospora* sp.

The results evidenced statistically significant variations on the mean positions of fungal taxa during the study period in both sites (Castro Marim: $p<0.001$, F=1.97; Ria de Aveiro: $p<0.05$, F=1.42). The one-way ANOVA performed for each fungal taxon revealed that the differences

were statistically significant ($p<0.05$) for all fungal taxa, except for *L. marina.* However, the relative mean position of each fungus on the plants seemed to be maintained, as well as the spatial pattern of occupancy along the vertical axis of the plant by different fungal taxa.

Salinity requirements of fungi

To confirm the salinity requirements for the isolated fungi with vertical distribution pattern on the standing plants in natural environment, a culture experiment was performed. Fifteen strains were randomly selected from the 57 isolated fungi from Castro Marim salt marsh representing 8 fungal taxa, namely *B. spartinae* (2 strains), *B. obiones* (2 teleomorph strains), *L. marina* (2 strains), *N. retorquens* (2 strains), *P. spartinicola* (2 strains), *Phoma* sp. 1 (2 strains), *S. orae-maris* (1 strain) and *Stagonospora* sp. (2 strains); 13 strains were selected from the 66 isolated fungi from Ria de Aveiro salt marsh representing 6 fungal taxa, specifically *B. obiones* (2 teleomorph strains and 2 anamorph strains), *Lulworthia* sp. (2 strains), *N. retorquens* (1 strain), *P. spartinicola* (2 strains), *Phoma* sp. 1 (2 strains) and *Stagonospora* sp. (2 strains).
The comparison between mycelia growth rates under two different culture conditions, on media lacking and containing diluted seawater, provided additional information about the ecological preferences of each taxon (Fig. 8).



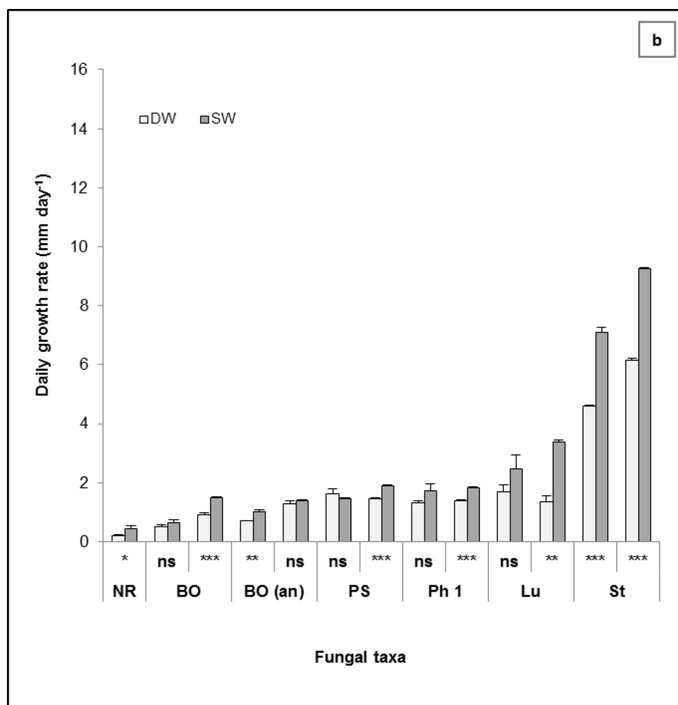


Fig. 8 Daily growth rate of eight fungal taxa isolated from Castro Marim (a) and six fungal taxa isolated from Ria de Aveiro (b) salt marshes under two culture conditions, media with diluted seawater *(SW)* and with distilled water *(DW)*: *BO Byssothecium obiones* (teleomorph), *BO(an) Byssothecium obiones* (anamorph), *BS Buergenerula spartinae*, *LM Leptosphaeria marina*, *Lu Lulworthia* sp., *NR Natantispora retorquens*, *Ph1 Phoma* sp. 1, *PS Phaeosphaeria spartinicola*, *SO Sphaerulina orae-maris*, *St Stagonospora* sp. The differences between growth rates were considered statistically significant (* p<0.05; ** p<0.01; *** p<0.001) or non-significant (ns, p>0.05)

The obtained results indicated that all the tested fungal strains grew in both culture conditions. Although some strains representing the same species exhibited contradictory growth results,

the majority of strains showed significantly higher growth under saline conditions. An exception was *L. marina*, which grew faster in media without seawater, and *P. spartinicola*, which grew equally well under both conditions. Additionally, the results revealed different growth rates among fungal taxa, with *Lulworthia* sp., *Stagonospora* sp. and *B. spartinae* displaying the highest growth rates on both media.

Discussion

Fungal diversity

In the present study, 23 fungal taxa were identified associated with early stages of decomposition of *S. maritima*; 20 fungal taxa inhabited Castro Marim salt marsh and 14 occurred in Ria de Aveiro salt marsh (Table 2). Both fungal communities were predominantly represented by the Ascomycota, particularly Dothideomycetes and Sordariomycetes. The clear preference and dominance of these taxonomic groups in intertidal habitats, characterized by alternate cycles of immersion and exposure, have been widely documented in several studies (e.g. Gessner and Kohmeyer 1976; Gessner 1977; Newell et al. 1989, 1996a, 2000a; Samiaji and Barlocher 1996; Alias and Jones 2000; Barata 2002; Al-Nasrawi and Hughes 2012).
The species richness and diversity of fungal communities colonizing *S. maritima* in Castro Marim and Ria de Aveiro salt marshes were similar to those found in the same host plant by Barata (2002, 2006), in *S. alterniflora* by Gessner (1977), Samiaji and Barlocher (1996) and Al-Nasrawi and Hughes (2012), and in *Spartina densiflora* Brongn. by Peña and Arambarri (1998). Both fungal communities from Castro Marim and Ria de Aveiro salt marshes were found to be well-balanced, without a clear dominance. These findings corroborated observations by Gessner et al. (2007) and Van Ryckegem et al. (2007), who denoted that fungal communities associated with *Spartina* spp. are not particularly complex, with a low diversity and few dominant species.

Fungal species composition of communities associated with Spartina spp.

A comparison between the species composition of the studied fungal communities associated with *S. maritima* and the list of marine fungi reported from *Spartina* species (Kohlmeyer and Kohlmeyer 1979; Barata 2002; Kohlmeyer and Volkmann-Kohlmeyer 2002; Calado and Barata 2012) revealed nine common fungal species. These species can be categorized into three groups according to their geographical distribution and substrate specificity: (1) host-genus-exclusive fungi that have been described from different *Spartina* species in different geographical locations, namely *Anthostomella spissitecta*, *B. spartinae*, *B. obiones*, *Mycosphaerella* sp. I and *P. spartinicola* (Gessner & Kohlmeyer 1976; Peña and Arambarri 1998; Barata 2002; Kohlmeyer and Volkmann-Kohlmeyer 2002; Cornick et al. 2005; Walker and

Campbell 2010); (2) temperate fungi that have a broad substrate preference, such as *L. marina* and *S. orae-maris*, which were found on *Spartina* spp. (Gessner and Kohlmeyer 1976; Cavaliere 1977; Kohlmeyer and Kohlmeyer 1979; Shoemaker and Babcock 1989; Peña and Arambarri 1998; Barata 2002) and also on *Juncus roemerianus* (Cavaliere 1977; Kohlmeyer and Kohlmeyer 1979) and driftwood (Cavaliere 1977; Kohlmeyer and Kohlmeyer 1979; Peña and Arambarri 1996; Figueira and Barata 2007); and (3) cosmopolitan fungal species that have also been recorded on a wide variety of substrates, such as *Aniptodera chesapeakensis* and *D. pelagicum* in temperate (Jones et al. 1998; Barata 2006; Figueira and Barata 2007) and tropical climates (Sadaba et al. 1995; Poon and Hyde 1998a; Hyde and Sarma 2006; Alias et al. 2010; Khan and Manimohan 2011; Manimohan et al. 2011).

In this study, *B. spartinae*, *B. obiones* and *P. spartinicola*, mentioned in the literature as the main colonizers of decaying *Spartina* plants (Gessner 1977; Newell and Wasowski 1995; Newell and Zakel 2000; Newell et al. 2000a; Newell 2001a; Barata 2002; Buchan et al. 2002, 2003; Cornick et al. 2005; Lyons et al. 2010; Walker and Campbell 2010), were also very frequent or frequent in both studied communities.

The high number of common fungal species colonizing different *Spartina* hosts corroborated the existence of a very stable core group of fungi, mainly dominated by the same host-exclusive fungi. This core group apparently is not much affected by variations in abiotic conditions (Gessner and Kohlmeyer 1976).

Different *Spartina* species, however, supported some different fungal species. This finding, previously demonstrated by Blum et al. (2004) and Lyons et al. (2010), was attributed to the higher variation in the morphology and chemical composition between different host species than within the same species. In fact, and as pointed out by several authors (Gessner and Kohlmeyer 1976; Torzilli et al. 2006), the substrate quality appears to be primarily responsible for determining fungal community composition. This reason could explain the absence of some fungal species that have been frequently collected from other *Spartina* species on *S. maritima* plants in this study and in a similar study performed by Barata (2002), as well as the exclusive presence of other fungal species in these communities.

In concordance with Barata (2002) study, *N. retorquens* was found to be the most frequent and dominant species in the two studied fungal communities, although it has not been reported from other *Spartina* species. The absence of *N. retorquens* on these host plants is not easy to explain, considering that this fungal species has been collected from driftwood in temperate regions (Jones et al. 1998; Figueira and Barata 2007; Azevedo et al. 2012) and from different substrates in tropical climates (Sadaba et al. 1995; Prasannarai and Sridhar 2001; Alias et al. 2010). However, it could be related with the fact that most of studies that inventoried *Spartina* spp. focused mainly or exclusively on leaf blades. Similarly, *P. viscosus*, another cosmopolitan species that has been described from temperate and tropical regions (Peña and Arambarri 1996; Jones et al. 1998; Prasannarai and Sridhar 2001; Figueira and Barata 2007; Alias et al. 2010), was reported for the first time on standing plants of *Spartina* by Barata (2002), on drift stems of the same host plant by Azevedo et al. (2012), and collected again in this study.

Even though the high overlapping of fungal communities associated with the same host species, it were found some variations in the mycota associated with standing plants of *S. maritima* in different salt marshes in terms of species composition and frequency patterns.

S. maritima was found to be a new host plant for seven fungal species; *A. spissitecta*, *C. obiones*, *D. gaudefroyi*, *L. marina* and *S. haliclysta* were exclusively collected from Castro Marim plants, *Halosarpheia trullifera* was exclusively present in Ria de Aveiro plants and *Camarosporium roumeguerii* occurred in both study sites (Table 2). From all the mentioned fungal species, only *A. spissitecta* and *L. marina* have been previously described from other *Spartina* species. *C. roumeguerii*, *C. obiones* and *D. gaudefroyi* have been observed inhabiting other salt marsh plants (Inderbitzin et al. 2002; Abdel-Wahab and Bahkali 2012). *S. haliclysta* and *H. trullifera* have been found colonizing the seaweed *Pelvetia canaliculata* (Abdel-Wahab and Bahkali 2012) and driftwood in temperate regions (Peña and Arambarri 1996; Jones et al. 1998), respectively. With the exception of *C. roumeguerii* and *L. marina*, all the other fungal species were also new records for Portugal.

Although the differences in sampling methods applied in this study and in Barata (2002) study may explain some differences between surveyed fungal communities, these are more likely to have resulted from different environmental conditions in the study sites. Similarly, this last reason could explain the differences in the fungal communities from Castro Marim and Ria de Aveiro salt marshes. The higher species richness and diversity found in Castro Marim salt marsh may be attributed to suitable environmental conditions given by a more preserved habitat; these conditions may favour the colonization and reproduction of less well-adapted species.

The fact that *S. maritima* community is more reduced and fragmented in Ria de Aveiro marsh and the vestigial presence of mercury in this study site (not measured in the present study, but mentioned by Coelho et al. 2009, 2014 and Pereira et al. 2009) might have provided less favourable conditions for colonization by occasional and infrequent species. The total mercury, as demonstrated by Coelho et al. (2009), accumulates more in old leaves than in stems, although in lower concentrations than in belowground biomass. These conditions seemed to have no effects on the most frequent fungal taxa in the community. The resistance of dominant saprobic ascomycetous fungi associated with *S. alterniflora* to several potentially toxic pollutants was already demonstrated by Newell and Wall (1998) and Newell et al. (2000b); they measured the living fungal biomass and sexual productivities of dominant fungi in standing-decaying leaf blades in the presence of mercury, methylmercury, polychlorinated biphenyls, chlorinated organocyclic insecticide toxaphene, chromium, copper, lead and polycyclic aromatic hydrocarbons and showed that these biological parameters were not affected by the presence of the toxicants. Moreover, the omnipresence of dominant fungal species in different *Spartina* communities of different states of conservation was also revealed by Cornick et al. (2005) – stable versus declining beds of *Spartina anglica* C. E. Hubbard – and Walker and Campbell (2010) – natural versus created *S. alterniflora* salt marshes.

However, the absence of *Mycospharella* sp. I in Castro Marim salt marsh, as well as the absence and infrequent occurrence of *L. marina* and *S. orae-maris* respectively in Ria de Aveiro salt marsh, was more difficult to interpret under an ecological perspective. The absence of *L. marina* from three salt marshes highly exposed to anthropogenic pressure surveyed by Barata (2002) and the absence of *S. orae-maris* from the most polluted one (Barata 2002) suggested that both species may require habitats with favourable conservation status to occur.
On the other hand, the slightly differences in the tidal regime in Castro Marim and Ria de Aveiro salt marshes, which reflected on the flooding patterns and salinity exposure in the study sites, might have limited the colonization by *L. marina*, *S. orae-maris* and *Mycosphaerella* sp. I. Although *S. maritima* plants occurred at a higher topographic level in Castro Marim salt marsh, they were shorter (±39 cm) and submerged more frequently during the sampling period (79% days per month); *S. maritima* plants in Ria de Aveiro salt marsh colonized a lower topographic level but were taller (±49 cm), being totally submerged less frequently (60% days per month). In addition to different flooding frequency, both study sites presented different salinity ranges, varying more in Castro Marim than in Ria de Aveiro salt marsh (Table 1).
Furthermore, and as pointed out by Torzilli et al. (2006) and Lyons et al. (2010), the intraspecific morphological variations in host plants, which implied differences in their chemical composition, may have restricted the colonization process to the more well-adapted species. Also, the general smaller size of leaf blades of *S. maritima* in Castro Marim might have promoted the interspecific competition among fungi, conditioning the colonization by *Mycosphaerella* sp. I. Newell and Zakel (2000) observed that *Mycosphaerella* sp. II tended to produce more ascospores in larger and thicker leaf blades.
Although these last enumerated hypotheses to explain the presence/absence of fungal species in the communities are merely speculative, this study clearly demonstrated the importance of environmental factors (biotic and abiotic) for the colonization of some fungal taxa, especially less frequent ones.

Vertical distribution patterns of fungi

The fungal taxa colonizing *S. maritima* plants were found to exhibit vertical distribution patterns on the host plants in Castro Marim and Ria de Aveiro salt marshes (Fig. 2). This finding is in concordance with similar studies that focused on fungal communities inhabiting mangroves trees and shrubs (Sadaba et al. 1995) and other standing grasses distributed from brackish (Poon and Hyde 1998a, b; Van Ryckegem and Verbeken 2005a, b; Van Ryckegem et al. 2007) to more saline tidal marshes (Gessner 1977; Kohlmeyer and Kohlmeyer 1979; Barata 2002; Al-Nasrawi and Hughes 2012). Fungal taxa occupy their own ecological niche, as a consequence of species-specific ecological requirements (i.e. chemical composition of the substrate, and temperature, salinity and moisture of the microhabitat) and interspecific competition (Jones 2000).

The separation of basal, middle or top portions of the plants in both study sites based on the distribution of fungal taxa (Figs. 3 and 4) emphasized the importance of micro-environmental conditions for the colonization and establishment of ecological niches. An integration of all results highlighted some ecological patterns, particularly of the most representative fungal taxa in the community (Figs. 2 and 4): *N. retorquens*, *B. obiones*, *Lulworthia* sp. and *L. marina* (in Castro Marim) occurred mostly in the more frequently flooded plant portions (basal portions) associated with stems and/or leaf sheaths; *B. spartinae*, *S. orae-maris* and *Phoma* sp. 1 occupied preferentially the middle portions, colonizing stems and/or sheaths and basal portions of leaf blades; *P. spartinicola*, *Stagonospora* sp. and *Mycosphaerella* sp. I (in Ria de Aveiro) were found in the less inundated top portion of the plants, mainly associated with leaf blades.

The less obvious separation between middle and top portions in Ria de Aveiro plants established by the cluster analysis (Fig. 3) might have resulted from differences in plant heights; the plants were considerable shorter in the first four sampling periods than in the remaining period (Fig. 7). As a consequence, the fungal communities that were mainly found on the top portions during the period June 2011–August 2012, were detected on the middle portion during the period October 2010–April 2011. Therefore, and considering the fact that the relative positions of fungi on the standing plants were maintained, it was not attributed any biological meaning for this result.

The fact that all fungal taxa occurred more frequently on the same plant portion of Castro Marim and Ria de Aveiro plants, i.e. basal, middle or top portion, even though the morphological differences between the host plants suggested a clear preference for the micro-environmental conditions of the colonized microhabitat. However, none of the fungal taxa was exclusively restricted to one particular microhabitat; in fact, the majority was observed in two plant portions, and only *P. spartinicola* and *Phoma* sp. 1 were detected in all portions in Castro Marim and Ria de Aveiro salt marshes. This finding, in addition to the differences in the absolute distribution ranges of common fungal taxa in plants from both salt marshes (Fig. 2) and the seasonal variation of vertical position of fungi on the plants (Fig. 7), suggested that the plant substrate might be the major key factor determining distribution boundaries. This reason may also explain the higher similarity between middle and top plant portions in Ria de Aveiro salt marsh based on the frequency of occurrence of fungal taxa harboured in those microhabitats (Fig. 4), which were found to be more similar in terms of the proportion of vegetative structures available for colonization.

The distribution ranges of fungal taxa, determined in this study by the vertical positions of fruiting structures on standing plants, were assumed to be more realistic for the species more frequently collected than for the infrequent ones. This assumption was complemented with the argument that a high density of fruiting structures implies substantial supportive matrix of an active mycelium (Newell and Porter 2000) to infer about the importance of the fungi on the decay process. Thus, it was hypothesized that fungal taxa that were producing more fruiting structures over a larger distribution area, such as *B. obiones* and *N. retorquens* on leaf sheaths and *P. spartinicola*, *Phoma* sp. 1 and *Stagonospora* sp. on leaf blades, were presumably

assuming a more active role in the decomposition of colonized plant tissues. However, the absence/paucity of fruiting structures does not directly indicate if a fungus is absent/less abundant on the substrate, but probably that the required species-specific biotic and abiotic conditions for reproduction were not achieved.

Some of the ecological niches revealed in the present study have already been documented in similar studies performed with *S. maritima* and also with other species of *Spartina*; specifically, the higher occurrence and dominance of *P. spartinicola* and *Mycosphaerella* sp. I in the top of the canopy on leaf blades (Gessner 1977; Newell and Wasowski 1995; Newell and Zakel 2000; Newell et al. 2000a; Newell 2001a; Barata 2002; Buchan et al. 2002, 2003; Lyons et al. 2010; Walker and Campbell 2010; Al-Nasrawi and Hughes 2012). Newell and Wasowski (1995) demonstrated that the extent of occupancy of fruiting structures on *S. alterniflora* produced by *P. spartinicola* is not affect by the frequency of flooding, but rather by the colonized vegetative structure, i.e. the lower percentage occupancy was found on the leaf sheaths. *B. spartinae* observed in present study and Barata (2002) study (as ascomata and hyphopodia) on leaf sheaths and stems in the middle portion of *S. maritima* plants has been mostly recorded on *Spartina* leaf blades and sheaths in the middle-top portions (Newell and Wasowski 1995; Newell et al. 2000a; Newell 2001a; Cornick et al. 2005; Walker and Campbell 2010; Al-Nasrawi and Hughes 2012). In addition to host exclusivity, the general agreement between this study and previous studies indicated that these fungi also presented a high degree of preference for vegetative structures and for particular vertical positions on standing plants.

The sequential vertical positions of *Lulworthia* sp., *N. retorquens*, *B. obiones*, *S. orae-maris*, *D. pelagicum*, *B. spartinae*, *Stagonospora* sp., *P. spartinicola* and *Phoma* sp. 1 along *S. maritima* plants described by Barata (2002) was confirmed in the present study, with slight variations. Even though there are similarities in the relative positions and colonized vegetative structures of common fungal taxa found in this study and Barata (2002) study, the absolute positions were different.

Thus, this study demonstrated that although the vertical distribution patterns of fungi resulted from the combined effect of micro-environmental conditions and substrate preference, it is this last biological factor that exerts a greater influence in determining the distribution range of these fungi.

Ecological characterization of fungi

Even though most of the fungal species recorded in this study are considered as obligate marine fungi by Jones et al. (2009), Barata (2002) presented some strong evidences to support the classification into obligate or facultative marine fungi. The higher or lower tolerance of fungi to salinity, air exposure and water submersion conditions that influences their vertical distribution on standing plants may, in fact, be related with their origin and physiological and morphological adaptations. In agreement with Barata (2002) observations, both fungal communities from Castro Marim and Ria de Aveiro salt marshes did not included terrestrial or

halotolerant fungi since the plants were normally totally submerged twice a day during high tides. Therefore, the results from the present study corroborate the classification of *Lulworthia* sp., *N. retorquens* and *B. obiones* into obligate marine fungi and *Stagonospora* sp. and *P. spartinicola* into facultative marine fungi, which were found on basal and top portions of the plants, respectively. *Lulworthia* sp. and *N. retorquens* were frequently collected by Barata (2006) from *S. maritima* baits exposed to permanent and temporary submersion conditions, which reinforce the argument that these fungi are highly adapted to marine environments. Moreover, Sadaba et al. (1995) also recorded *N. retorquens* on basal portions of *Acanthus ilicifolius*, an herbaceous mangrove standing plant.

The average vertical positions and distribution ranges of obligate and facultative fungi were taken into account to establish a virtual threshold value to distinguish from other fungal taxa. The threshold value (22 cm) was found to be situated in the middle plant portion, which means that this microhabitat constituted a vertical transition area for obligate and facultative marine fungi. As a transition zone, this microhabitat was colonized by fungal taxa both of the basal and top plant portions, which led to the greatest fungal richness, number of occurrences and diversity in both study sites (Table 3).

With this assumption, the fungal species more frequently recorded on basal portions, such as *P. viscosus* and *L. marina*, and on top portions, such as *Mycosphaerella* sp. I, *Mycosphaerella* sp. 2 and *S. haliclysta*, are likely to be obligate and facultative marine fungi, respectively.

The classification of the fungal taxa located in the middle plant portions was, though, more complicated. Nevertheless, and considering the established threshold value, the present study confirmed the classification of *S. orae-maris* as an obligate marine fungus and *D. pelagicum*, *B. spartinae* and *Phoma* sp. 1 as facultative marine fungi proposed by Barata (2002). Moreover, the results suggested that *C. obiones*, *D. gaudefroyi* and *Phoma* sp. 2, which occurred in the middle portion of Castro Marim plants, are facultative marine fungi.

Although the low occurrence of some fungal taxa in both salt marshes did not enable to distinguish their real distribution range, the presence of *A. chesapeakensis*, *H. trullifera* and *A. spissitecta* on lower plant portions and of *Fusarium* sp., *Leptosphaeria* sp. and *C. roumeguerii* on top plant portions might indicate that these species are obligate and facultative marine fungi, respectively. *A. spissitecta* was also found in lower portions of *Spartina* plants, being classified by Kohlmeyer and Volkmann-Kohlmeyer (2002) as an obligate marine fungus. *H. trullifera* was more frequently recorded by Jones and Kuthubutheen (1989) on submerged mangrove wood, which suggested that this species is, indeed, an obligate marine species.

A focus on the morphology of reproductive structures and mechanism of spores dispersal of the fungal taxa present along the vertical axis of the host plants seemed to corroborate the distinction previously made. As pointed out by Hyde and Lee (1995), Alias and Jones (2000) and Hyde and Sarma (2006), the subcommunities inhabiting the basal and top plant portions possessed, in general, morphological characteristics that well adapt them to marine and terrestrial environments respectively. The group of marine fungi that colonized the basal microhabitat included fungal taxa with membranous (e.g. *N. retorquens*), carbonaceous (e.g. *B.*

obiones) and coriaceous (e.g. *Lulworthia* sp.) ascomata, whereas the majority of the fungal taxa that occurred on the upper plant portions produced coriaceous ascomata, i.e. more resistant to desiccation imposed by a terrestrial habitat. Regarding ascus morphology and spore-discharge mechanism, the Sordariomycetes with dissolving unitunicate asci and passive spore-discharge dominated the basal portions, while the Dothideomycetes with bitunicate asci and an active spore-discharge inhabited the top portions. These findings are in agreement with Fell and Newell (1998), Alias and Jones (2000), Barata (2002) and Hyde and Sarma (2006) studies. According with Kohlmeyer and Kohlmeyer (1979), the spore dispersal mechanism through a forceful ejection has probably evolved in terrestrial habitats, whereas a passive release of spores directly in water is more likely to have evolved in aquatic species, given the spores are easily washed away by tidal currents. The hypothesis that the active mechanism for spores discharge has a terrestrial origin was also proposed by Jones and Kuthubutheen (1989) referring to some mangrove fungi. No clear correspondence was found between the vertical position of fungal taxa on the plants and the colour, presence/morphology of spore appendages and position of reproductive structures on the plant tissues (i.e. immersed, erumpent, superficial); most of the fungal reproductive structures were immersed on the substrate.

The evidences showed in this study supported the existence of obligate and facultative marine fungi colonizing different positions on intertidal standing plants, with distinct morphological adaptations and possibly distinct origins.

Seasonality

The results revealed that fungal composition of the communities of Castro Marim and Ria de Aveiro salt marshes did not considerably change during the study period, with the most frequent fungi present in all sampling periods. This finding, which is in agreement with previous studies conducted in intertidal ecosystems (Buchan et al. 2003; Torzilli et al. 2006; Walker and Campbell 2010), reinforced the observation of Gessner (1977) of a characteristic, resilient and stable community associated with *Spartina* species.

The occurrences and production of fruiting structures by frequent and very frequent fungal taxa in both communities, though, varied over the sampling time, except for *N. retorquens* in Ria de Aveiro salt marsh (Figs. 5 and 6). In general, the variations in the frequencies of occurrence of fungal taxa and of high density of fruiting structures produced by the same species did not follow a regular pattern. For this reason, these variations cannot be directly related with the seasonal variations of temperature and humidity or inclusively with the seasonal variation of nitrogen content in decaying vegetative structures of *Spartina* plants (Newell 2001b; Cartaxana and Catarino 2002).

However, the reduction in the frequency of occurrence of *P. spartinicola* and *Stagonospora* sp. in the two communities during the warmer periods suggested an effect of seasonality on the life cycle of these fungal species. The climatic factors also seemed to have affected the production of fruiting structures by the same species and *N. retorquens* in Castro Marim salt marsh. The

interference of seasonality in the life cycle of fungal species and particularly the general decrease of fungal biomass and productivity during the warmest months have been already demonstrated in previous studies (Samiaji and Barlocher 1996; Castro and Freitas 2000; Newell and Porter 2000; Newell 2001b). Newell (2001a) documented higher percentages of released spores for *P. spartinicola* during cooler seasons. In contrast, Buchan et al. (2003) study revealed that the abundance of *P. spartinicola* did not change with the seasonality. The differences between these two studies could be related with the applied methodologies.

The lack of obvious seasonal patterns pointed to a requirement of longer studies to better discriminate the effect of seasonality in fungal community dynamics and avoid biased conclusions. The variations in the vertical distribution of most frequent fungal taxa during the study period seemed not to be directly related with seasonality but either with the phenological growth patterns of the host plants.

Salinity requirements of fungi

The results from the culture experiment demonstrated that *B. obiones*, *B. spartinae*, *Lulworthia* sp., *N. retorquens*, *L. marina*, *P. spartinicola*, *Phoma* sp. 1, *S. orae-maris* and *Stagonospora* sp. grew on media lacking and containing seawater (Fig. 8), which suggests that there is not an absolute requirement of sodium chloride at concentrations found in seawater for growth. However, the growth rates in the two culture media were, in general, statistically different and higher under saline conditions, even for fungal taxa previously classified into facultative marine fungi, such as *B. spartinae*, *Phoma* sp. 1 and *Stagonospora* sp. The results from this experiment are in agreement with reported observations in similar studies (Jones and Jennings 1964; Sguros and Simms 1964; Jones 2000; Masuma et al. 2001; De la Cruz et al. 2006; Huang et al. 2011; Jones 2011; Pang et al. 2011; Burgaud et al. 2013) that marine fungi were capable of growing vegetatively without marine salts, although they generally exhibit an optimal growth under higher concentrations of salinity. The ability to grow without marine salts and tolerate salinity fluctuations likely confers an adaptive and competitive ecological advantage over their terrestrial counterparts in intertidal habitats subjected to intermittent dilution by freshwater inputs (Sguros and Simms 1964), i.e. seasonal precipitation and continuous freshwater discharges from adjacent rivers.

The fluctuations in salinity were demonstrated by some studies, though, to interfere in the production of antimicrobial metabolites by fungal species (Masuma et al. 2001; Huang et al. 2011) and in their sporulation (Jones 2011).

Even though the general tendency of fungal taxa to grow better in the presence of marine salts, two fungal species revealed different vegetative growth patterns. *P. spartinicola* demonstrated a higher physiological plasticity than the other fungal taxa to adapt to different culture media conditions, being able to grow to the same extent on media with and without sea salts; this behavior under culture conditions reinforced its classification into facultative marine fungi. *L. marina* was the only species showing a better growth on culture media without marine salts,

which contradicted the observations made on the field; the interpretation of its response was not straightforward since there is no additional evidence that this fungal species colonizes other less saline habitats.

The fact that the results from the culture experiment did not totally corroborate the field observations, suggests that it is not possible to distinguish obligate from facultative marine fungi based exclusively on vegetative growth responses. This means that is not recommended to apply the current definition of marine fungi in laboratory context, as argued by Kohlmeyer (1974), even with the certainty that all tested fungi are active in the community.

However, this experiment was important to demonstrate the high physiological plasticity and versatility of marine fungal taxa to adapt to different abiotic conditions, as well as species-specific salinity requirements. The differences among vegetative growth rates and particularly the faster growth of *Lulworthia* sp., *B. spartinae* and *Stagonospora* sp. (Fig. 8) may indicate that these fungal taxa have, in fact, high growth rates or, alternatively, that they were exploring more efficiently this particular artificial substrate.

Final remarks

This study, conducted in a less surveyed geographical region, supported the existence of a stable core group of fungi associated with *Spartina* species. Besides being dominated by the same host-exclusive ascomycetous fungi, the studied fungal communities also included other saprobic fungi exclusive to *S. maritima*, and seven new records were documented for this host plant and five for Portugal. This study also confirmed the species-specific vertical distribution patterns of fungi along the standing plants, which were attributed mainly to the substrate availability and to a lesser extent, the micro-environmental conditions of the habitat. The most frequent fungal taxa in the two communities revealed a high tolerance to salinity fluctuations and exhibited wide vertical distribution ranges and a high investment in the production of fruiting structures. These findings suggested that these fungal species were well-established and adapted to the intertidal habitat, exploring efficiently the substrate and consequently assuming an important and active key role in the early stages of decomposition of *S. maritima*.

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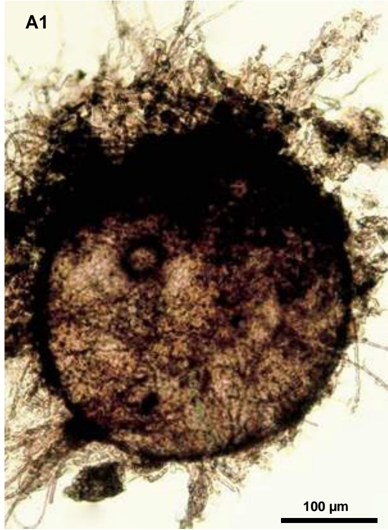
Supplemental Figure:

Diversity and ecological characterization of sporulating higher filamentous marine fungi associated with *Spartina maritima* (Curtis) Fernald in two Portuguese salt marshes; Microbial Ecology; Maria da Luz Calado*, Luís Carvalho, Ka-Lai Pang, Margarida Barata; * - Centre for Ecology, Evolution and Environmental Changes (Ce3C; Faculty of Sciences of University of Lisbon*,* Edifício C2, 5º Piso, Campo Grande, 1749-016 Lisboa, Portugal, Tel.: +351 217500577; Fax: +351 217500028), Department of Plant Biology (Faculty of Sciences of the University of Lisbon, Edifício C2, 2º Piso, Campo Grande, 1749-016 Lisboa, Portugal, Tel.: +351 217500047; Fax: +351 217500048), mdcalado@fc.ul.pt.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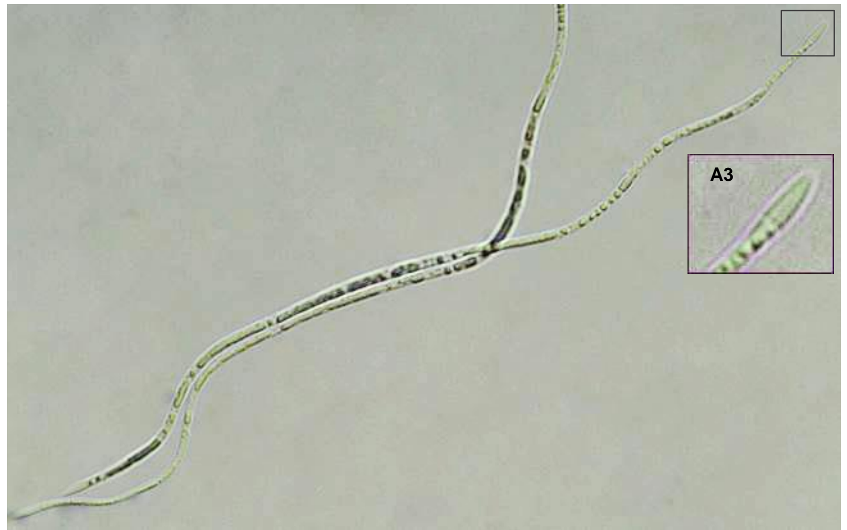
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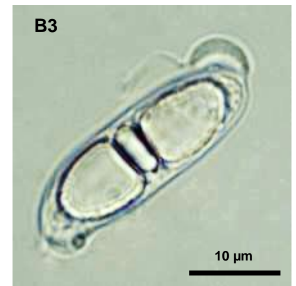
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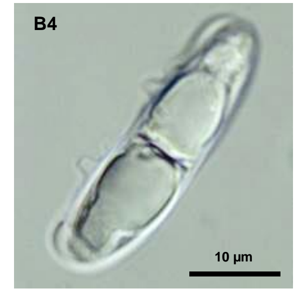
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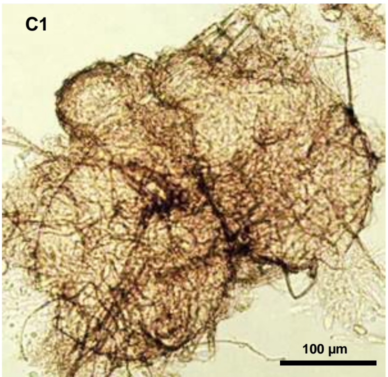
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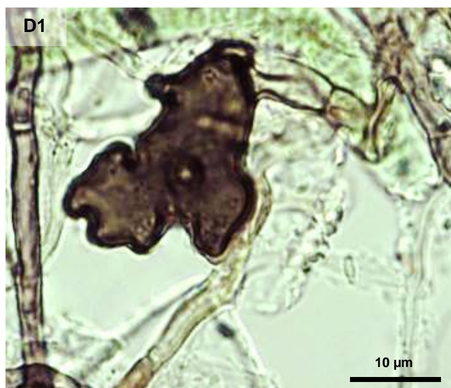
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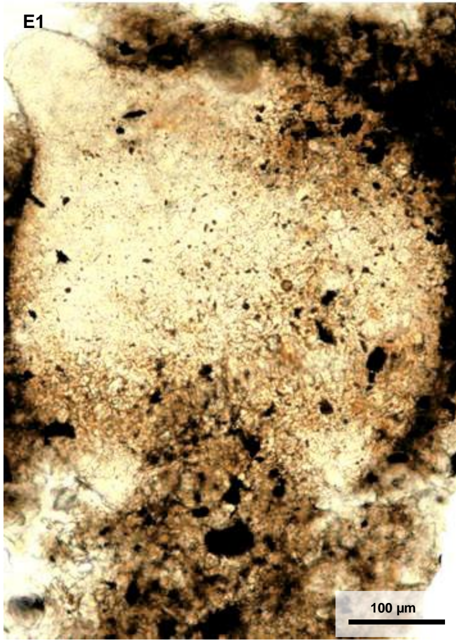
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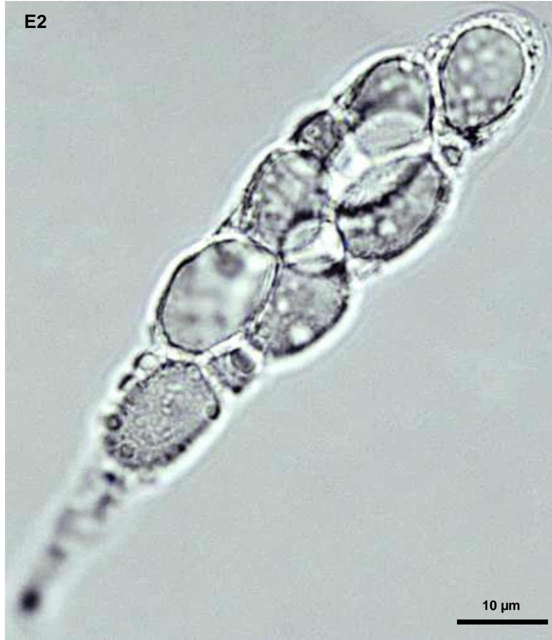
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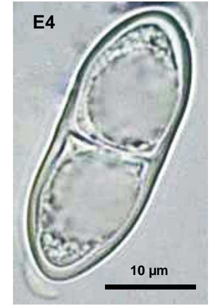
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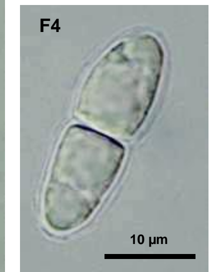
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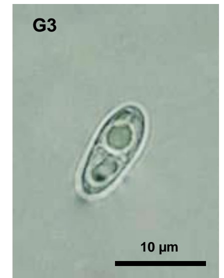
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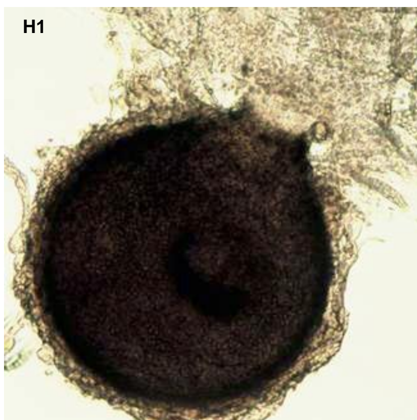
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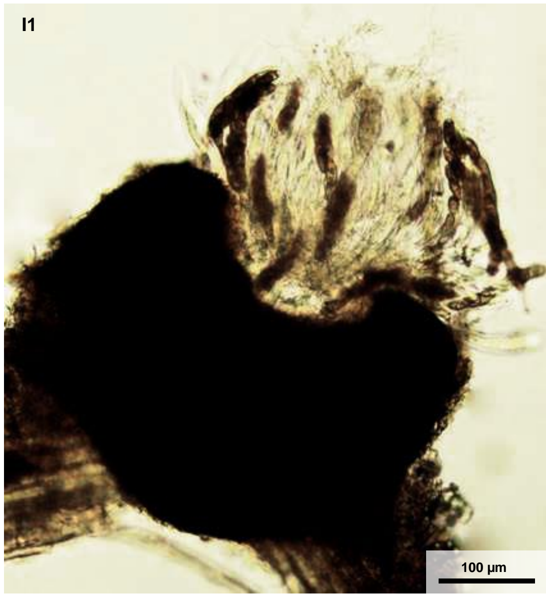
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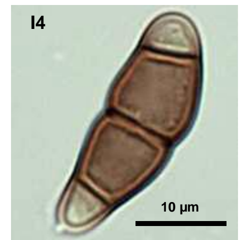
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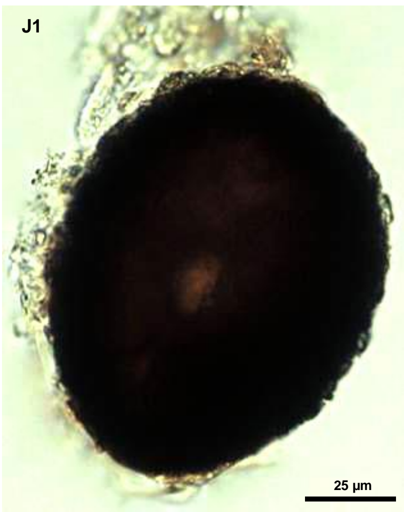
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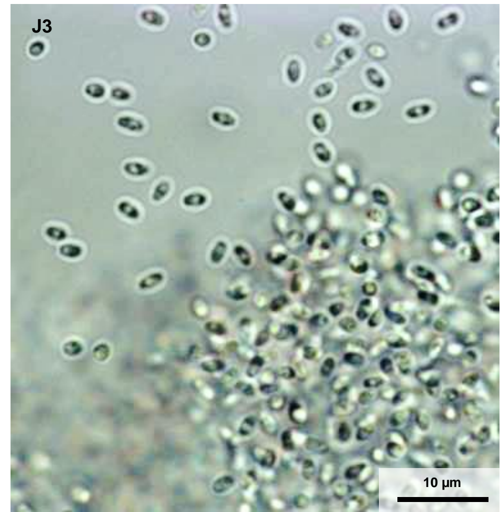
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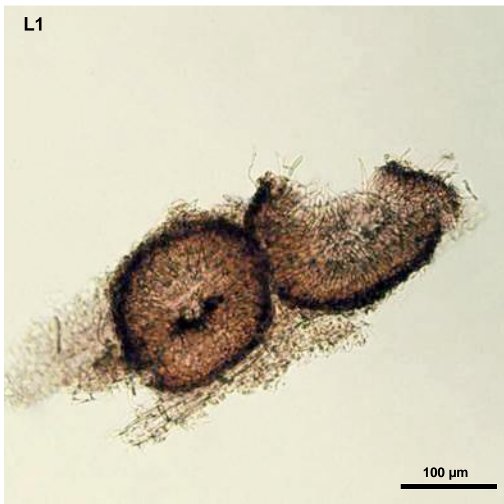
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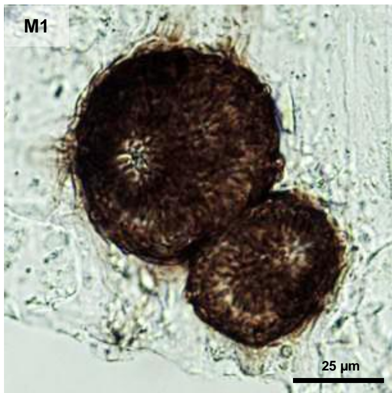
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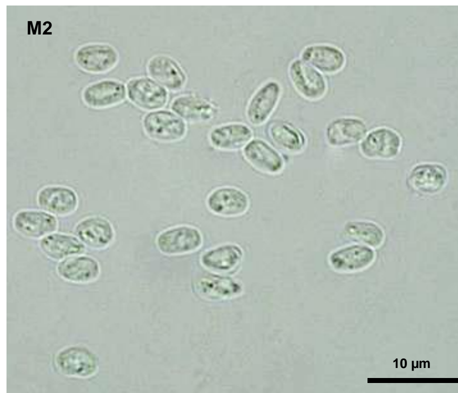
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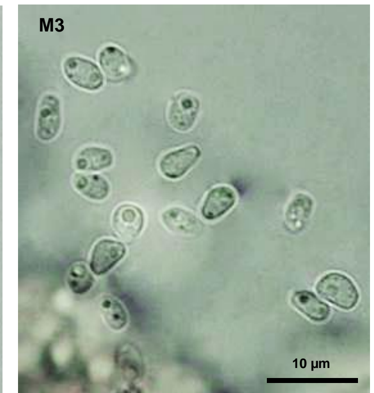
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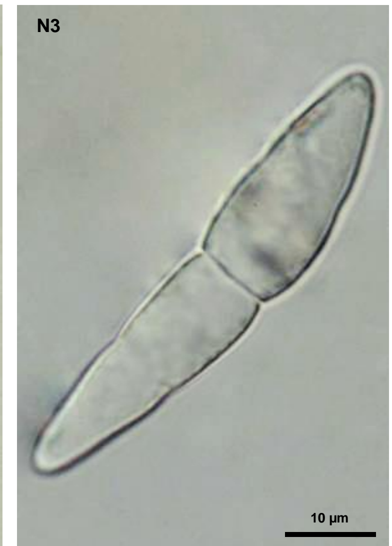
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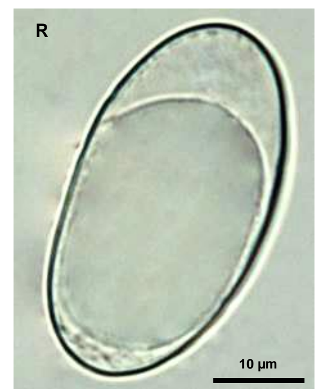
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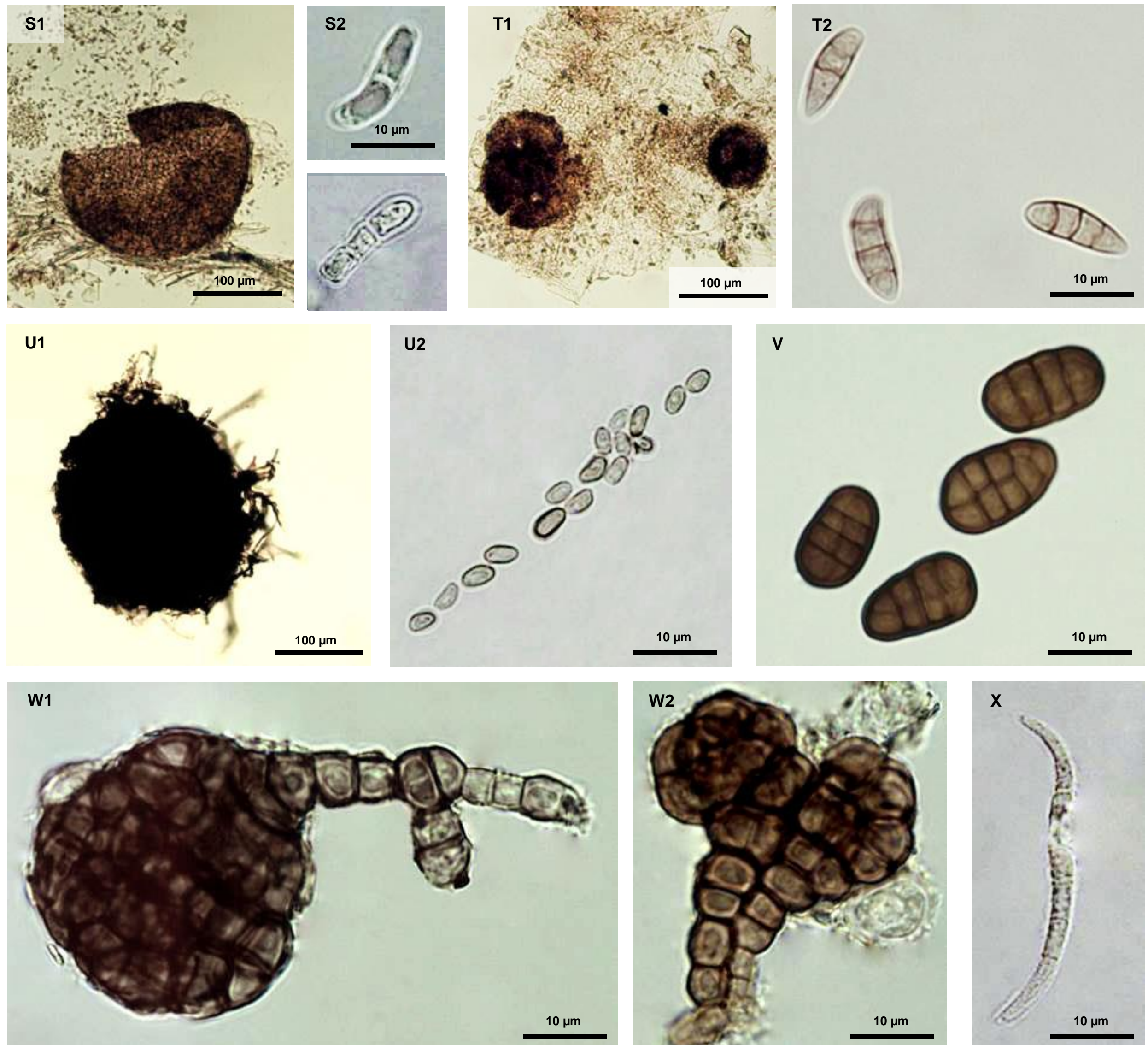

Fig. S1 Marine fungal taxa associated with *Spartina maritima* standing plants: *Lulworthia* sp. (A1-ascoma; A2-ascospores; A3- detail of the apical-chamber of ascospores); *Natantispora retorquens* (B1-ascoma; B2-immature ascus; B3/B4-ascospores); *Panorbis viscosus* (C1-ascomata; C2-ascus; C3-ascospores); *Buergenerula spartinae* (D1-hyphopodia; D2-ascus; D3/D4-ascospores); *Aniptodera chesapeakensis* (E1-ascoma; E2-immature ascus; E3/E4-ascospores); *Mycosphaerella* sp. I (F1-ascoma; F2-ascus; F3/F4-ascospores); *Mycosphaerella* sp.2 (G1-ascoma; G2-asci; G3-ascospore); *Sphaerulina orae-maris* (H1-ascoma; H2/H3-asci; H4-ascospore); *Byssothecium obiones*, teleomorph (I1-ascoma; I2-ascus; I3/I4-ascospores); *Byssothecium obiones*, anamorph (J1-pycnidium; J2-phialides; J3-conidia); *Phaeosphaeria spartinicola* (K1-ascoma; K2-asci; K3-ascospore); *Stagonospora* sp. (L1-pycnidia; L2/L3-conidia); *Phoma* sp.1 (M1-pycnidia; M2/M3-conidia); *Leptosphaeria marina* (N1-ascoma; N2-ascus; N3-ascospore); *Leptosphaeria* sp. (O1-ascoma; O2-ascospore); *Decorospora gaudefroyi* (P-ascospore); *Anthostomella spissitecta* (Q-ascospore); *Halosarpheia trullifera* (R-ascospore); *Phoma* sp.2 (S1-pycnidium; S2/S3-conidia); *Stagonospora halyclista* (T1-pycnidia; T2-conidia); *Coniothyrium obiones* (U1-pycnidium; U2-conidia); *Camarosporium roumeguerii* (V-conidia); *Dictyosporium pelagicum* (W1/W2-conidia); *Fusarium* sp. (X-conidium).

CHAPTER 3 – Ecological preferences of marine fungi associated with standing decaying plants of *Spartina maritima* (Curtis) Fernald

2016, in revision for new submission

Ecological preferences of marine fungi associated with standing decaying plants of *Spartina maritima* (Curtis) Fernald

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Abstract

Fungal communities inhabiting live, senescent and decaying leaf sheaths, stems and leaf blades of standing plants of *Spartina maritima* (Curtis) Fernald in two Portuguese salt marshes were assessed by fruiting structures- and *ITS* sequence-based identification methods. Forty-five fungi were found on early decaying plants. The molecular method enabled identification of infrequent ascomycetes and basidiomycetes (filamentous and yeasts), and the asexual morph of *Byssothecium obiones* and *Phaeosphaeria halima*. The results suggested that the occurrence and ecological role of most frequent fungi on different plant substrates depend on the phase of plant life cycle, micro-environmental conditions of substrates and potential fungal competitors. Specifically, *B. obiones*, *Natantispora retorquens* and *Lulworthia* sp. 1 seem to be involved in the decay of lower culms; *Mycosphaerella* sp. I, of leaf blades; *P. halima* and *Stagonospora* sp. 1, of upper standing leaves; and *Buergenerula spartinae* and *Phaeosphaeria spartinicola*, of all vegetative structures. The presence of these fungi on live vegetative structures suggested an earlier endophytic colonisation.

Keywords

Marine fungi; *Spartina maritima*; early stages of decay; leaf sheaths; leaf blades; stems; ecological preferences; potential ecological roles; *ITS* rDNA libraries

Introduction

Spartina species represent one of the main dominant halophytes in temperate salt marsh ecosystems, contributing significantly to the annual biomass production and nutrient budget (Castillo et al. 2010; Curado et al. 2013). Given the fact that the bulk of *Spartina* biomass is composed of cell wall recalcitrant polymers, particularly lignocellulose (≈ 75%; Maccubbin and Hodson 1980; Hodson et al. 1984; Torzilli and Andrykovitch 1986), the release of nutrients to the surrounding environment strictly depends on the degradative activity of microbial communities, particularly of saprobic fungi (Gessner 1980; Torzilli and Andrykovitch 1986; Bergbauer and Newell 1992). The decomposition of *Spartina* species, similarly with other grass-like plants, is initiated in senescent vegetative structures that remain in natural position (Newell et al. 1989; Newell 1993; Castro and Freitas 2000; Newell and Porter 2000; Menéndez and Sanmartí 2007). The senescence, which may begin very early in the growing season (Samiaji and Barlocher 1996; Menéndez and Sanmartí 2007), occurs gradually from the outer and lower vegetative structures towards the inner and higher structures (Samiaji and Barlocher 1996; Kneib et al. 1997; Newell 2001a). Typically, a sequential abscission of the more decomposed leaf blades occurs, but the leaf sheaths remain attached to stems until complete breakdown (Healy and Walters 1994; Menéndez and Sanmartí 2007). The stems, enwrapped by

successive and overlapping layers of leaf sheaths that arise from each node, only start to senesce when become more exposed to air, i.e. when the surrounding leaf sheaths are fragmented or detached from the stems.

The senescent process is induced by external and internal factors and involves physiological changes and chemical transformation of the plant tissues (Buchanan-Wollaston 1997; Hortensteiner and Feller 2002). Although most antimicrobial and more complex polymers remain after senescence (Valiela et al. 1979; Wilson et al. 1986a,b; Graça et al. 2000), some soluble tannins and phenolics may leach out quickly during the initial stages of decay process (Raghukumar 2004). Decomposition is initiated by mycelial fungal species that previously inhabit internal photosynthetic plant tissues as endophytes and/or are able to withstand the antimicrobial substances in the senescent material (Raghukumar 2004; Cornick et al. 2005). Some endophytic fungal species demonstrated physiological adaptability to switch to a saprobic life style after a physical or maturational change in the host plants, like senescence (Alva et al. 2002; Kumaresan and Suryanarayanan 2002; Promputtha et al. 2007, 2010). Among these, some endophytes were shown to be morphologically and phylogenetically similar with their saprobic counterparts (Alva et al. 2002; Kumaresan and Suryanarayanan 2002; Promputtha et al. 2007, 2010).

Pioneer mycelial decomposers combine their filamentous and pervasive growth mode with the production of extracellular enzymes to initiate an extensive lysis with physical disruption and chemical transformation of intact plant tissues (Newell et al. 1996b; Hyde et al. 1998; Newell and Porter 2000; Kis-Papo 2005). Simultaneously, some pioneer fungi have the ability to metabolize some antimicrobial substances, such as cinnamic acids, ameliorating microenvironmental conditions for subsequent fungal decomposers (Newell 1993). Ascomycetous fungi play a more relevant role during this initial standing-decay phase, dominating the microbial assemblages (Newell 1993, 1996, 2001a; Newell and Porter 2000). As the decomposition carried out by these fungi proceeds, the organic matter content in the various vegetative structures decreases (Buchan et al. 2003); a considerable fraction of dissolved organic matter is readily leached out, and about 10–20% is converted into fungal biomass in form of mycelia and reproductive structures (Newell et al. 1989, 1996a, 2000; Newell and Wasowski 1995; Samiaji and Barlocher 1996; Newell and Porter 2000; Newell 2001b). The substrate remains potentially nutrient-depleted with high lignin and low nitrogen contents in final stages of decomposition (Torzilli and Andrykovitch 1986). The physical and chemical changes of *Spartina* substrates during decomposition have been postulated to interfere with fungal community dynamics (Gessner 1977; Buchan et al. 2003; Raghukumar 2004; Barata 2006).

Previous studies on the decomposition process of *Spartina* species mainly focused on the dominant fungi associated with standing decaying leaf blades of *S. alterniflora* in U.S. salt marshes (Newell and Wasowski 1995; Newell and Zakel 2000; Newell et al. 2000; Newell 2001a; Buchan et al. 2002; Lyons et al. 2010; Walker and Campbell 2010).

The fungal community associated with *Spartina maritima* (Curtis) Fernald, one of the main pioneer colonisers of salt marsh mudflats in southern European and North African coastlines

(Sánchez et al. 2001) and one of the dominant macrophytes in Portuguese salt marshes, has been poorly investigated: Barata (1997, 2002) and Calado et al. (2015) together inventoried fungi on standing plants from five geographically and physically distinct salt marshes; Barata (2006) surveyed stem baits exposed to different abiotic conditions in one of these salt marshes; and Azevedo et al. (2012) analysed drift stems collected in four Portuguese sandy beaches. The identification of species relied on the morphological features of fungal structures produced at the time of collection and/or after incubation.

The advent of molecular techniques has contributed greatly to overcome the major drawbacks of traditional culture- and microscopy-based techniques in documenting fungal diversity and to provide a more accurate taxonomic classification of fungi (Buchan et al. 2002; Lyons et al. 2005; Pang and Mitchell 2005; Torzilli et al. 2006; Walker and Campbell 2010; Jones 2011a; Abdel-Wahab and Bahkali 2012). Internal transcribed spacers (*ITS*) of the rDNA operon region have been frequently used for fungal species identification because they are present in multiple copies within each cell, easily amplifiable by Polymerase Chain Reaction (PCR) and highly variable in sequence (Buchan et al. 2002; Nilsson et al. 2006; Arnold 2007; Aveskamp et al. 2010; Schoch et al. 2012; Velmurugan et al. 2013). Except for some taxonomic groups which have very conserved *ITS*, this region is widely accepted as the most powerful universal DNA barcode for fungi (Schoch et al. 2012; Toju et al. 2012). Even though many marine fungal species have been already sequenced, *ITS* sequences for a number of marine fungi are not yet available in international databases for sequence comparison (Nilsson et al. 2006; Porter and Golding 2011; Schoch et al. 2012). For this reason, and also the systematic biases associated with PCR amplification and/or cloning techniques (Buchan et al. 2002, 2003; Pang and Mitchell 2005; Toju et al. 2012), molecular techniques should be coupled with other methods to study fungal community dynamics.

Thus, the present study intended to provide a more realistic representation of fungal community associated with standing plants of *S. maritima* in salt marsh ecosystems, by combining morphological and molecular approaches. Specifically, the main purposes were to complement the list of fungi associated with *S. maritima* recently published by Calado et al. (2015) and to infer about ecological preferences and involvement of these fungi during the decay process of standing leaf sheaths, leaf blades and true stems, using morphological (fungal structures identification) and molecular (*ITS* sequencing) techniques.

Material and Methods

Study sites, host plant and sampling procedure

The study was performed in two salt marshes located in distinct geographical regions, the Guadiana estuary (Castro Marim) situated in the southeastern coast (37.23° N, 7.42° W), and

the Ria de Aveiro coastal lagoon in the northwest of Portugal (40.62° N, 8.74° W). Both estuary and coastal lagoon also differ in physical configuration and conservation status.

Field sampling procedure involved a bimonthly collection of mature standing plants in Castro Marim and Ria de Aveiro salt marshes, over a two-year period (October 2010 to August 2012). Twenty plants were collected on the first three sampling periods and 15 plants afterwards (a total of 390 plants); five additional plants during the second sampling year were also collected for isolation of marine fungi.

A detailed description of the study sites, *S. maritima* communities and sampling criteria can be found in Calado et al. (2015).

Morphology-based species identification

The collected plants were carefully rinsed with running tap water to remove fine-grained sediments and seaweeds, and air-dried. Each air-dried plant was first separated into three categories according to the vegetative structure –leaf blade, leaf sheath and stem – and subsequently into three further subcategories based on the visually observed physiological state of the structure – live (green parts), senescent (yellow parts, with the physical structure still intact) and decaying (brown parts, with the physical structure visibly altered). This separation resulted in nine different samples that were designated as substrates, i.e. live leaf sheaths, senescent leaf sheaths, decaying leaf sheaths, live stems, senescent stems, decaying stems, live leaf blades, senescent leaf blades and decaying leaf blades.

Fungal structures (fruit bodies, spores and hyphopodia) observed on each plant substrate were picked up under a dissecting microscope (Wild M8), mounted in a drop of sterile seawater on a slide, examined under a light microscope (Leitz Laborlux S, with Normaski) with detailed morphology recorded. The fungi were identified using the dichotomous keys of Kohlmeyer and Kohlmeyer (1979), Kohlmeyer and Volkmann-Kohlmeyer (1991), Hyde and Sarma (2000) and Jones et al. (2009).

Plant substrates collected in the same study site and sampling date were mixed together, frozen at -80 °C and freeze-dried for three days in a Christ Alpha I-5 apparatus at 10^{-1}mbar and -42 °C.

Isolation of marine fungi and preservation of pure cultures

Several strains of the most frequent fungal taxa were isolated by single spore method (described in detail by Calado et al. 2015). Each pure culture was maintained in active growth in cornmeal agar made with sterile aged diluted seawater (CMA/sw 50 %) at 4 °C, and as mycelial discs (7 mm diameter) in McCartney bottles filled with sterile diluted seawater (50%) at 4 °C and in cryotubes filled with glycerol (10%) at -80 °C.

DNA extraction and amplification of ITS genes

Twenty-three fungal isolates (belonging to the nine most frequently collected fungal taxa; Calado et al. 2015) obtained from *S. maritima* were used to create a reference database for comparing and identifying fungal sequences recovered from 88 lyophilized plant samples (representing the first sampling year, October 2010 to August 2011).

Genomic DNA was extracted from axenic cultures using a standard phenol–chloroform protocol adapted from Pang et al. (2008). The DNA extraction from plant samples involved the maceration of c.a. 0.05 g of each plant sample to a fine powder in liquid nitrogen and a thermal incubation of the material (water bath at 70 °C for 15 min, liquid nitrogen for 2 min and water bath at 70 °C for 15 min); the subsequent steps of the procedure were identical to the procedure of DNA extraction from axenic cultures.

Amplification and sequencing of the *ITS* regions of pure fungal cultures were conducted using primer pairs ITS5 (5´ GGAAGTAAAAGTCGTAACAAGG 3´)/ ITS4 (5´ TCCTCCGCTTATTGATATGC 3´) (White et al., 1990). The primer pairs ITS1-F_KYO1 (5´ CTHGGTCATTTAGAGGAASTAA 3´)/ ITS4_KYO3 (5´CTBTTVCCKCTTCACTCG 3´) (Toju et al., 2012) were used for selective amplification of fungal rDNA in mixed DNA samples (plant materials). Both primer pairs cover partial sequences of *18S* rRNA, complete sequences of *ITS1*, *5.8S* rRNA and *ITS2*, and partial sequences of *28S* rRNA genes.

PCR amplifications were performed in 25 µL reaction volumes, containing 0.8 µM of each primer, 12.5 µL of Taq Premix (Cat.No. RT803A, Bioman, New Taipei City, Taiwan) and 1 µL of genomic DNA. PCR was carried out on a thermocycler (Biometra T3000) using the following parameters: initial denaturation for 5 min step at 95 °C, 34 cycles of 30 s at 95 °C (denaturation), 30 s at 55 °C (primer annealing) and 30 s at 72 °C (elongation), then a final elongation step of 5 min at 72 °C.

Amplicons of pure fungal isolates were directly sent to Genomics Biosci. & Tech. (New Taipei City, Taiwan) for sequencing, using the same pair of PCR primers.

Amplicons from plant samples were cloned in order to isolate individual amplicons of the mixed PCR products; in this study, cloning and restriction analysis were preferred over the next-generation sequencing approach given the expected high number and lengths of the amplicons.

ITS region clone libraries construction and restriction profiles analysis

PCR products from each plant sample were purified using the EasyPure PCR/Gel Extraction Kit (BIOMAN, Scientific CO, LTD, Taiwan), following the manufacturer's instructions. Purified amplicons were ligated into a TA cloning vector, using the RBC TA Cloning Vector Kit (RBC Bioscience Corp., Taiwan). After ligation, the mixture was used directly to transform *Escherichia coli* DH5α competent cells using RBC HIT Competent Cells Kit (RBC Bioscience Corp., Taiwan). Detection of transformed cells with recombinant DNA was performed through blue-white screening technique. Twenty white colonies were selected, and each one was transferred

into Luria Broth medium; a colony PCR was conducted in 20 µL reaction volume, with 2 µL of every bacterial suspension, 0.5 µM of each previously used primers and 10 µL of Taq Premix. *ITS* region clone libraries from 88 plant samples included a total of 1037 sequences. A restriction fragment length polymorphism (RFLP) analysis was used to categorise phylogenetically related sequences (Pang and Mitchell 2005).

Several restriction enzymes were tested on *ITS* of pure fungal isolates of the most common fungi on *S. maritima* and HpyF31 (DdeI) produced patterns that enabled differentiation of different fungal species (results not shown). Restriction enzyme digestion reaction followed the manufacturer´s instructions (Cat.No. ER1881, Thermo Scientific, New Taipei City, Taiwan). All the clones that exhibited different restriction profiles in the same plant sample were selected. Three hundred out of 1037 clones were sent to Genomics Biosci. & Tech. (New Taipei City, Taiwan) for sequencing in one direction.

Sequence alignment and phylogenetic analyses

ITS sequences of the 23 pure fungal isolates were assembled and manually adjusted to obtain a consensus sequence using DNA Baser Sequence Assembler v4.10 software (2014; Heracle BioSoft, www.DnaBaser.com). These sequences, as well as the 300 single-stranded full-length sequences retrieved from plant samples, were compared with those sequences available in GenBank database of National Center for Biotechnology Information (NCBI). The fungal *ITS* sequence-based classification relies on the phylogenetic relationships among species, using the BLAST best-hit method to classify an unknown sequence (Porter and Golding 2011). In this study, the sequences were identified to lowest possible taxonomic level based on a cut-off value of 97% of sequence similarity and 90% of sequence cover for species proposed by Nilsson et al. (2012) and Blaalid et al. (2013). Identification name provided by reference database was always adopted over the one provided by public database whenever there was a higher homology between the unknown sequence and a reference sequence. All the BLAST best-hits were retrieved from GenBank for further analysis. Even though BLAST top hits were mostly selected, the next best hits were retrieved whenever there was a better proposal in the list with similar cover/identity rates, i.e. of a fungal species that had been previously identified by morphological methods but not isolated in culture.

In a preliminary approach, all the sequences assigned to the same taxonomic families were grouped together, aligned by ClustalW and submitted to a phylogenetic maximum-parsimony analysis using Molecular Evolutionary Genetics Analysis (MEGA) v.6.06 software (Tamura et al. 2013); operational taxonomic units (OTUs) were then defined. One representative sequence was randomly chosen from each defined OTU; 62 out of 300 clones were selected and sent to Genomics Biosci. & Tech. (New Taipei City, Taiwan) for sequencing the complementary strand.

The sequences of representative clones, isolates and BLAST hits representing the main taxonomic classes were automatically aligned by ClustalW and refined manually with ambiguous regions of alignment removed using MEGA v.6.06 software.

Phylogenetic and neighbor-joining distance analyses were additionally performed using the same software, in order to confirm the OTUs previously established. Phylogenetic trees were constructed using maximum-parsimony and neighbor-joining methods. Maximum-parsimony trees were performed using 100 heuristic searches with random stepwise addition of sequences, tree-bisection-reconnection (TBR) branch swapping algorithm and maximum number of trees to retain set to 100. Characters were equally weighted and gaps were treated as missing data. Neighbor-joining trees were performed using p-distance, Jukes-Cantor, Kimura-2 and Logdet parameter substitution models. Topological robustness of the phylogenetic trees in both maximum-parsimony and distance analyses was estimated by performing 1000 bootstrap replicates. Neighbor-joining distance analysis was based on a pairwise-distance model.

Data analyses

A more accurate representation of species richness in different plant substrates from Castro Marim and Ria de Aveiro salt marshes was obtained by combining the results provided by morphological and molecular methods from the first-sampling year. Since the number of fungal taxa recorded on each plant substrate might represent a downward-biased estimator of the total species richness (Gotelli and Colwell 2010), species richness in the same substrates was also calculated using Chao 2 and Jacknife 2 estimators ($S_{Chao2} = S_{obs} + \left(\frac{m-1}{m}\right)\frac{Q1\ (Q1-1)}{2\ (Q2+1)}$ and $S_{Jack2} = S_{obs} + \left[\frac{Q1(2m-3)}{m} - \frac{Q2(m-2)^2}{m(m-1)}\right]$, where S_{obs} is the total observed number of fungal taxa, m the number of samples, $Q1$ the number of fungal taxa that occurred in only one sample, and $Q2$ the number of fungal taxa that occur in two samples); according to Gotelli and Colwell (2010), these estimators are more appropriate to replicated presence/absence data, correcting the observed species richness based on the frequencies of the very rarest species. A sample-based rarefaction was not performed because one of the plant substrates included only two samples.

Species composition of fungal communities inhabiting different plant substrates of Castro Marim and Ria de Aveiro salt marshes were also compared based on presence/absence and percentage frequencies of occurrence of fungal taxa in communities. Presence/absence of fungal taxa detected by morphological and/or molecular methods on each plant substrate (live, senescent and decaying leaf sheaths, stems and leaf blades), sampling period and study site during the first sampling year was compiled in two dataset matrices (one matrix per each study site). Percentage frequencies of occurrence of fungal taxa identified by morphological method on each plant substrate, sampling period and study site during the total sampling period were also determined and organized in two complementary dataset matrices (one matrix per each study site); percentage frequencies of occurrence of fungi were obtained from the number of plants in which a specific fungus occurred on each substrate divided by the total number of plants x 100. Contrary to the general studies, the binary data (presence/absence) were herein more informative than frequency data because they included the results provided by two

different but complementary methods; moreover, they did not depend on the fruiting patterns of fungi, and attributed the same weight to frequent and infrequent species. Nevertheless, percentage frequencies of occurrence of fungi on plant substrates throughout the 2-year study period provided robust information on fruiting preferences of marine fungi.

Given the complexity of datasets, multivariate analyses were carried out using the PAST v2.17c statistical software (Hammer et al. 2001) to reduce and summarize the data.

A preliminary and exploratory Cluster analysis was performed on sample-by-species matrices with binary data using Bray-Curtis similarity measure index. This index is suggested as an ideal coefficient to be used for the construction of similarity matrices given its effectiveness in dealing with datasets containing multiple blocks of zeros (Rees et al. 2004). The values identified as outliers were excluded from further analyses.

Additionally to the Cluster analysis, a Nonmetric multidimensional scaling (MDS) analysis was also applied on the reconstructed dataset matrices. MDS analysis, which is one of the most widely accepted ordination techniques in microbial ecology, only displays the samples in the plot based on values generated in a similarity matrix (Clarke and Warwick 2001; Rees et al. 2004; Ramette 2007). Another ordination method, a Detrended Correspondence Analysis (DCA), was also performed with relative frequency data in order to confirm patterns in dimensional coordinate plots; DCA is appropriate to model relative frequency data, enabling a simultaneous visualization of the samples and species in the ordination plot along an environmental gradient. Bray-Curtis index was selected for MDS analysis, whereas chi-squared index was implicit in DCA.

Differences between a priori groups (i.e. plant substrates) established on the basis on the presence/absence of species in the samples were statistically assessed through a one-way ANOSIM. A post hoc test to assess the significance of pairwise comparisons between substrates was also carried out.

Results and Discussion

Molecular identification of ITS sequences retrieved from plant samples

The majority of the 1037 *ITS* sequences recovered from *S. maritima* plant samples (99%) were identified. As demonstrated previously by Toju et al. (2012), the primers pair ITS1-F_KYO1/ ITS4_KYO3 used in this study was highly fungal-specific, considering the low number of non-fungal sequences recovered with these primers (0.1%); in contrast, most of the sequences amplified by the primers pair ITS5/ ITS4 belonged to *S. maritima* (86%).

The restriction enzyme HpyF31 (DdeI) was demonstrated to be able to provide better discriminatory results for fungal sequences than HaeIII, frequently used in T-RF technique (Buchan et al. 2002; Walker and Campbell 2010).

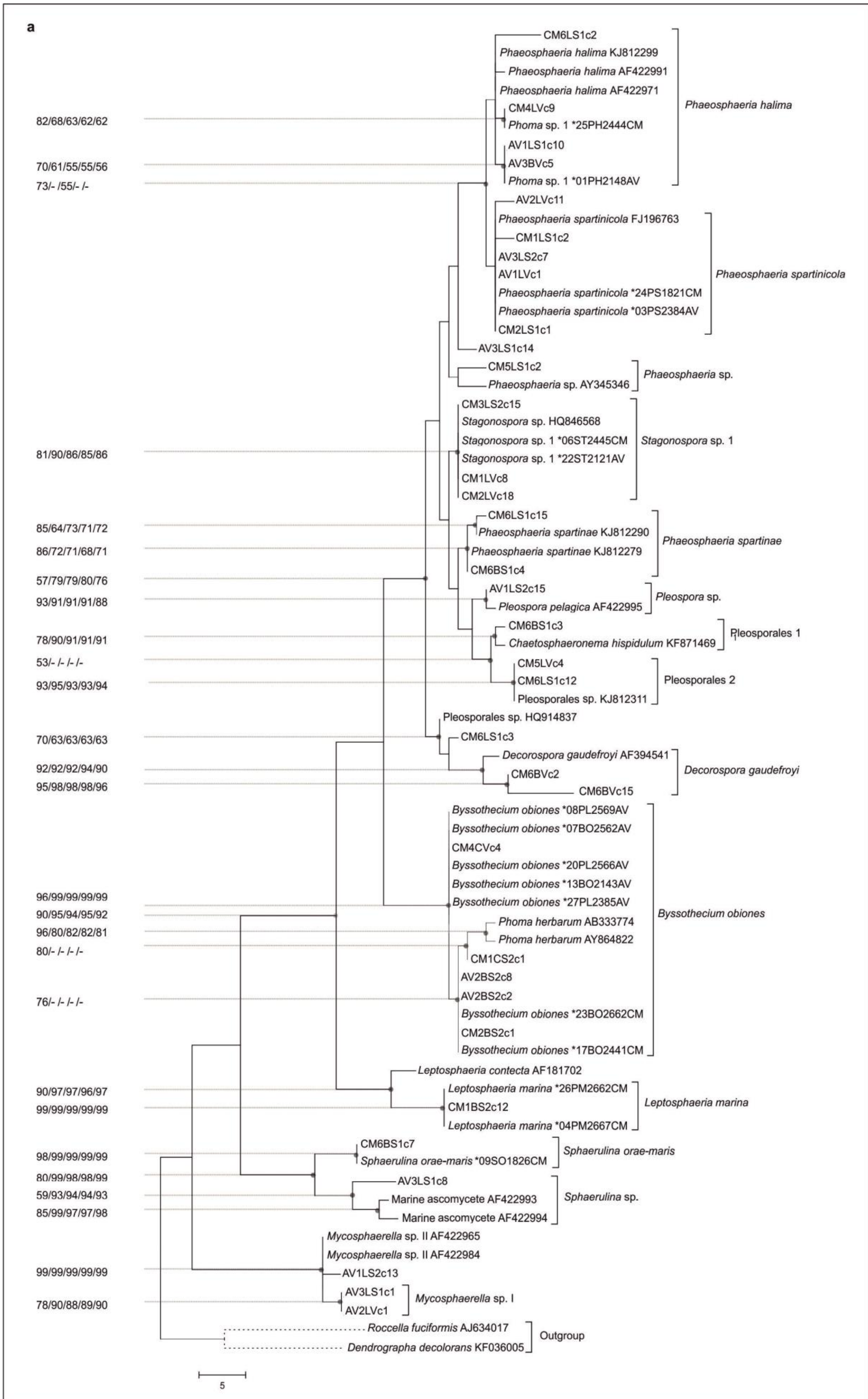
Fungal *ITS* sequence length (including partial 18S and 28S and full length 5.8S rDNA) of the representative clones from both Castro Marim and Ria de Aveiro salt marshes ranged from 365 to 674 bp. These sequences were identified to the lowest taxonomic rank possible and grouped in OTUs based on results from BLAST searches and phylogenetic analyses (Table 1, Figs. 1a, b).

Table 1 Fungal taxa identified on *Spartina maritima* samples by molecular methods. Pure fungal cultures previously isolated by morphological methods and representative clones assigned to the same fungal species on the basis of similar BLAST results and positions on phylogenetic trees, were grouped together. Best BLAST hits of clones and pure fungal isolates are mentioned, as well as homology scores between sequences

	Pure fungal isolates (i) and clones (c) codes	Accession number	BLAST best-hits	Cover/ Identity (%)	Identified fungal taxa
					Ascomycota; Dothideomycetes
i	*Byssothecium obiones* *07AV2562	KX263858	*Phoma herbarum* (AB333774)	100/ 92	*Byssothecium obiones* (P. Crouan & H. Crouan) M.E. Barr
i	*Byssothecium obiones* *08AV2569	KX263859	*Phoma herbarum* (AB333774)	100/ 92	
i	*Byssothecium obiones* *13AV2143	KX263860	*Phoma herbarum* (AB333774)	100 /92	
i	*Byssothecium obiones* *17CM2441	KX263861	*Phoma herbarum* (LN827678)	93/ 94	
i	*Byssothecium obiones* *20AV2566	KX263862	*Phoma herbarum* (AY864822)	100/ 92	
i	*Byssothecium obiones* *23CM2662	KX263863	*Phoma herbarum* (LN827678)	92/ 94	
i	*Byssothecium obiones* *27AV2385	KX263864	*Phoma herbarum* (AY864822)	100/ 92	
c	AV2BS2c2	KX263805	*Phoma herbarum* (AY864822)	100/ 93	
c	AV2BS2c8	KX263806	*Phoma herbarum* (AY864822)	100/ 94	
c	CM1CS2c1	KX263807	*Phoma herbarum* (AY864822)	99/ 96	
c	CM2BS2c1	KX263808	*Phoma herbarum* (AY864822)	100/ 92	
c	CM4CVc4	KX263809	*Phoma herbarum* (AY864822)	100/ 94	
c	CM6BVc2	KX263813	*Decorospora gaudefroyi* (AF394541)	91/ 91	*Decorospora gaudefroyi* (Pat.) Inderb., Kohlm. & Volkm.-Kohlm.
c	CM6BVc15	KX263814	*Decorospora gaudefroyi* (AF394541)	90/ 89	
i	*Leptosphaeria marina* *04CM2667	KX263865	*Leptosphaeria contecta* (AF181702)	88/87	*Leptosphaeria marina Ellis & Everh.*
i	*Leptosphaeria marina* *26CM2662	KX263866	*Leptosphaeria contecta* (AF181702)	89/ 87	
c	CM1BS2c12	KX263820	*Leptosphaeria contecta* (AF181702)	93/ 88	
c	AV2LVc1	KX263826	*Mycosphaerella* sp.II (AF422984)	82/ 93	*Mycosphaerella* sp. I
c	AV3LS1c1	KX263827	*Mycosphaerella* sp.II (AF422965)	90/ 89	
c	AV1LS2c13	KX263825	*Mycosphaerella* sp. II (AF422965)	84/ 90	*Mycosphaerella* sp. 3
i	*Phaeosphaeria halima* *01AV2148	KX263872	*Phaeosphaeria halima* (KJ812299)	87/ 97	*Phaeosphaeria halima* (T.W. Johnson) Shoemaker & C.E. Babc.
i	*Phaeosphaeria halima* *25CM2444	KX263873	*Phaeosphaeria halima* (AF422991)	100/ 99	
c	AV1LS1c10	KX263831	*Phaeosphaeria halima* (AF422971)	95/ 97	
c	AV2LVc11	KX263832	*Phaeosphaeria halima* (AF422971)	95/ 96	
c	AV3BVc5	KX263833	*Phaeosphaeria halima* (AF422991)	96/ 97	
c	CM4LVc9	KX263834	*Phaeosphaeria halima* (AF422991)	100/ 99	
c	CM6LS1c2	KX263835	*Phaeosphaeria halima* (AF422991)	98/ 94	
c	CM6BS1c4	KX263837	*Phaeosphaeria spartinae* (KJ812279)	97/ 100	*Phaeosphaeria spartinae* (Ellis & Everh.) Shoemaker & C.E. Babc.
c	CM6LS1c15	KX263838	*Phaeosphaeria spartinae* (KJ812290)	97/ 99	

i	*Phaeosphaeria spartinicola* *03AV2384	KX263874	*Phaeosphaeria spartinicola* (FJ196763)	100/ 98	*Phaeosphaeria spartinicola* Leuchtm.
i	*Phaeosphaeria spartinicola* *24CM1821	KX263875	*Phaeosphaeria spartinicola* (FJ196763)	94/ 100	
c	AV1LVc1	KX263839	*Phaeosphaeria spartinicola* (FJ196763)	94/ 98	
c	AV3LS2c7	KX263840	*Phaeosphaeria spartinicola* (FJ196763)	94/ 98	
c	CM1LS1c2	KX263841	*Phaeosphaeria spartinicola* (FJ196763)	94/ 98	
c	CM2LS1c1	KX263842	*Phaeosphaeria spartinicola* (FJ196763)	100/ 98	
c	CM5LS1c2	KX263836	*Phaeosphaeria* sp. (AY345346)	97/ 90	*Phaeosphaeria* sp.
c	AV1LS2c15	KX263843	*Pleospora pelagica* (AF422995)	66/ 96	*Pleospora* sp.
c	CM6BS1c3	KX263844	*Chaetosphaeronema hispidulum* (KF871469.1)	85/ 91	Pleosporales 1
c	CM5LVc4	KX263845	Pleosporales (KJ812311)	94/ 99	Pleosporales 2
c	CM6LS1c12	KX263846	Pleosporales (KJ812311)	94/ 99	
c	CM6LS1c3	KX263847	Pleosporales (HQ914837)	97/ 98	Pleosporales 3
i	*Sphaerulina orae-maris*	KX263876	Marine ascomycete (AF422994)	92/ 82	*Sphaerulina orae-maris* Linder
c	CM6BS1c7	KX263848	Marine ascomycete (AF422993)	88/ 83	
c	AV3LS1c8	KX263849	Marine ascomycete (AF422993)	99/ 90	*Sphaerulina* sp.
i	*Stagonospora* sp. 1 *06CM2445	KX263877	*Septoriella phragmitis* (NR132926)	100/ 96	*Stagonospora* sp. 1
i	*Stagonospora* sp. 1 *22AV2121	KX263878	*Septoriella phragmitis* (NR132926)	100/ 96	
c	CM1LVc8	KX263850	*Stagonospora* sp. (HQ846568)	97/ 96	
c	CM2LVc18	KX263851	*Stagonospora* sp. (HQ846568)	100/ 98	
c	CM3LS2c15	KX263852	*Stagonospora* sp. (HQ846568)	96/ 96	
c	AV3LS1c14	KX263853	*Stagonospora* sp. (HQ846568)	92/ 94	*Stagonospora* sp. 2
					Ascomycota; Sordariomycetes
c	CM6BS1c2	KX263802	*Anthostomella brabeji* (EU552098)	79/ 90	*Anthostomella* sp.
i	*Buergenerula spartinae* *05CM2661	KX263856	*Buergenerula spartinae* (AF422962)	100/ 99	*Buergenerula spartinae* Kohlm. & R.V. Gessner
i	*Buergenerula spartinae* *14CM2122	KX263857	*Buergenerula spartinae* (AF422962)	100/ 100	
c	AV4BVc2	KX263803	*Buergenerula spartinae* (AF422962)	100/ 100	
c	CM6BVc13	KX263804	*Buergenerula spartinae* (AF422961)	99/ 99	
c	CM3BS2c1	KX263811	*Cosmopora* aff. *viliuscula* (JN995627)	99/ 93	*Cosmospora* sp.

c	AV3LVc6	KX263816	*Fusarium oxysporum* (KJ082096)	100/ 98	*Fusarium oxysporum* Schltdl.
c	AV3CVc6	KX263818	_		*Halosarpheia* sp.
i	*Lulworthia* sp. 1 *10AV2385	KX263867	*Lulwoana* sp. (FJ430723)	84/ 84	*Lulworthia* sp. 1
i	*Lulworthia* sp. 1 *21AV2389	KX263868	*Lulwoana* sp. (FJ430723)	84/ 84	
c	AV1BS1c1	KX263821	*Lulwoana* sp. (FJ430722)	80/ 83	
c	AV5BVc12	KX263822	*Lulwoana* sp. (FJ430723)	84/ 84	
c	CM4CS1c3	KX263823	*Lulwoana* sp. (KF719969)	79/ 84	*Lulworthia* sp. 2
c	CM4CS1c15	KX263824	*Lulwoana* sp. (FJ430722)	78/ 85	
i	*Natantispora retorquens* *11CM2581	KX263869	*Panorbis viscosus* (AF422979)	70/ 97	*Natantispora retorquens* (Shearer et J.L. Crane) J. Campb., J.L. Anderson & Shearer
i	*Natantispora retorquens* *15AV2125	KX263870	*Panorbis viscosus* (AF422979)	72/ 97	
i	*Natantispora retorquens* *16AV2385	KX263871	*Panorbis viscosus* (AF422979)	70/ 97	
c	AV5BS2c2	KX263828	*Panorbis viscosus* (AF422979)	65/ 97	
c	AV5BVc7	KX263829	*Panorbis viscosus* (AF422979)	71/ 97	
c	AV3BVc7	KX263854	*Savoryella appendiculata* (HQ446350)	67/ 85	Unidentified Halosphaeriaceae 1
c	CM3BS2c4	KX263855	*Savoryella* sp. (HQ446362)	96/ 80	Unidentified Halosphaeriaceae 2
					Ascomycota; Eurotiomycetes
c	AV6LVc15	KX263830	*Penicillium chrysogenum* (HQ026745)	100/ 100	*Penicillium chrysogenum* Thom
					Ascomycota; Leotiomycetes
c	CM2BVc3	KX263817	Uncultured *Gloeotinia* (KF225810)	100/ 99	*Gloeotinia* sp.
					Basidiomycota; Agaricomycetes
c	AV3BVc1	KX263810	*Ceriporia lacerata* (KF850375)	97/ 99	*Ceriporia lacerata* N. Maek., Suhara & R. Kondo
c	AV3BVc15	KX263819	*Junghuhnia crustacea* (JN710554)	100/ 94	*Junghuhnia* sp.
					Basidiomycota; Tremellomycetes
c	CM4BVc2	KX263812	*Cryptococcus mangaliensis* (FJ008052)	100/ 99	*Cryptococcus mangalensis* Fell, Statzell & Scorzetti
					Basidiomycota; Cystobasidiomycetes
c	AV3BVc9	KX263815	*Erythrobasidium hasegawianum* (AF444522)	98/ 98	*Erythrobasidium hasegawianum* Hamam., Sugiy. & Komag.



a
CM6LS1c2
Phaeosphaeria halima KJ812299
Phaeosphaeria halima AF422991
Phaeosphaeria halima AF422971
CM4LVc9
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AV1LS1c10
AV3BVc5
Phoma sp. 1 *01PH2148AV
Phaeosphaeria halima
AV2LVc11
Phaeosphaeria spartinicola FJ196763
CM1LS1c2
AV3LS2c7
AV1LVc1
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Phaeosphaeria spartinicola *03PS2384AV
CM2LS1c1
Phaeosphaeria spartinicola
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CM5LS1c2
Phaeosphaeria sp. AY345346
Phaeosphaeria sp.
CM3LS2c15
Stagonospora sp. HQ846568
Stagonospora sp. 1 *06ST2445CM
Stagonospora sp. 1 *22ST2121AV
CM1LVc8
CM2LVc18
Stagonospora sp. 1
CM6LS1c15
Phaeosphaeria spartinae KJ812290
Phaeosphaeria spartinae KJ812279
CM6BS1c4
Phaeosphaeria spartinae
AV1LS2c15
Pleospora pelagica AF422995
Pleospora sp.
CM6BS1c3
Chaetosphaeronema hispidulum KF871469
Pleosporales 1
CM5LVc4
CM6LS1c12
Pleosporales sp. KJ812311
Pleosporales 2
Pleosporales sp. HQ914837
CM6LS1c3
Decorospora gaudefroyi AF394541
CM6BVc2
CM6BVc15
Decorospora gaudefroyi
Byssothecium obiones *08PL2569AV
Byssothecium obiones *07BO2562AV
CM4CVc4
Byssothecium obiones *20PL2566AV
Byssothecium obiones *13BO2143AV
Byssothecium obiones *27PL2385AV
Phoma herbarum AB333774
Phoma herbarum AY864822
CM1CS2c1
AV2BS2c8
AV2BS2c2
Byssothecium obiones *23BO2662CM
CM2BS2c1
Byssothecium obiones *17BO2441CM
Byssothecium obiones
Leptosphaeria contecta AF181702
Leptosphaeria marina *26PM2662CM
CM1BS2c12
Leptosphaeria marina *04PM2667CM
Leptosphaeria marina
CM6BS1c7
Sphaerulina orae-maris *09SO1826CM
Sphaerulina orae-maris
AV3LS1c8
Marine ascomycete AF422993
Marine ascomycete AF422994
Sphaerulina sp.
Mycosphaerella sp. II AF422965
Mycosphaerella sp. II AF422984
AV1LS2c13
AV3LS1c1
AV2LVc1
Mycosphaerella sp. I
Roccella fuciformis AJ634017
Dendrographa decolorans KF036005
Outgroup
82/68/63/62/62
70/61/55/55/56
73/- /55/- /-
81/90/86/85/86
85/64/73/71/72
86/72/71/68/71
57/79/79/80/76
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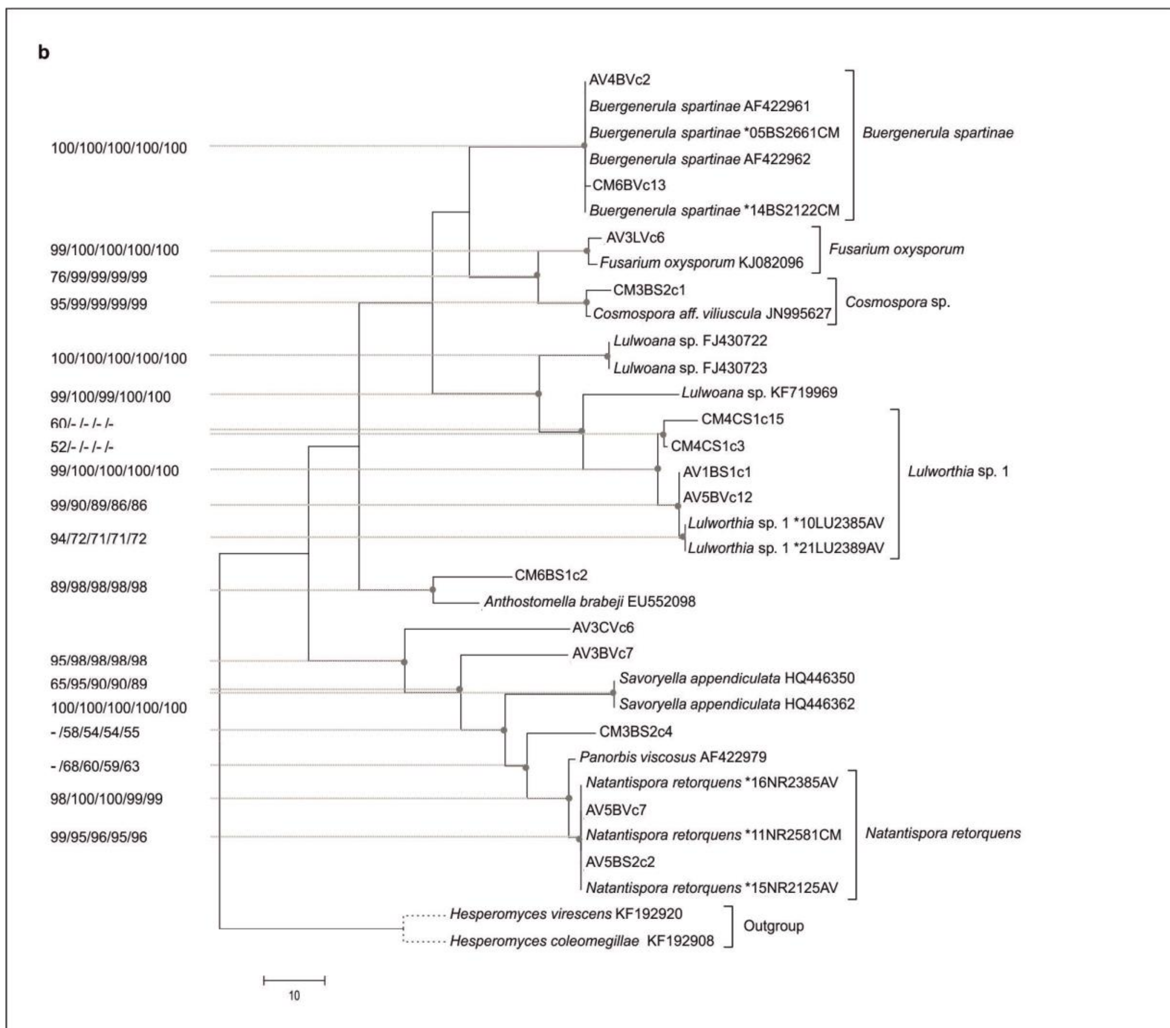


Fig. 1 Phylogenetic consensus trees based on a maximum parsimony analysis for the Dothideomycetes (a) and Sordariomycetes (b), showing the placement of clone sequences, isolate sequences (marked with an asterisk) and BLAST best-hit sequences. Bootstrap values higher than 50 are shown on the left side of the tree nodes; the first value refers to maximum parsimony analysis and the second and subsequent values to neighbor-joining analyses performed with *p*-distance, Jukes-Cantor, Kimura-2 and Logdet parameter substitution models respectively

Primer sets used in this study enabled amplification and species delimitations from both Ascomycota and Basidiomycota phyla (Table 1).

Maximum parsimony analyses of the *ITS* sequences included in the Dothideomycetes and Sordariomycetes generated 1 and 60 most-parsimonious trees respectively. The topological differences between the 60 equally parsimonious trees recovered from the Sordariomycetes dataset were restricted to branching order of clades and positions of three sequences (AV3BVc7, CM3BS2c4, CM4CS1c3).

Most of the terminal clades provided by parsimony and distance analyses were concordant and supported by similar high levels of bootstrap (Figs. 1a, b).

The best parsimony tree of the Dothideomycetes clearly separated different OTUs in different clades.

Clones AV1LS2c13, AV2LVc1 and AV3LS1c1 were identified as *Mycosphaerella* sp. based on the BLAST results (Table 1) and the highly-supported clade where they were integrated (Fig. 1a); however, the pairwise distance (9%) between sequences distinguished AV2LVc1 and AV3LS1c1 from AV1LS2c13. Even lacking an isolate for comparison, the high abundance of the first two amplicons in the clone libraries suggested that these sequences might belong to *Mycosphaerella* sp. I; this species was frequently identified on *S. maritima* plants from Ria de Aveiro salt marsh by its fruiting structures (Calado et al. 2015), but did not grow in culture medium.

Clones CM6BS1c7 and AV3LS1c8 clustered together on a high-supported clade with the isolate of *Sphaerulina orae-maris* and with marine ascomycetes AF422993 and AF422994 (Fig. 1a); the separation of this clade in two subclades and the high pairwise distance between the sequences of the clones (15%), suggested that this clade may represent two different species.

Clone CM1BS2c12 formed a monophyletic clade with isolates of *Leptosphaeria marina* (Fig. 1a). Also the clones CM1CS2c1, CM4CVc4, CM2BS2c1, AV2BS2c2, AV2BS2c8 and isolates of *Byssothecium obiones* grouped in a distinct monophyletic clade with a high bootstrap support (Fig. 1a). The integration of the isolates *08AV2569, *20AV2566 and *27AV2385 (previously misidentified as *Phialophorophoma litoralis* by morphological methods) and the isolates of *B. obiones* *23CM2662, *17CM2441, *13AV2143, *07AV2562 in the same highly supported clade (Fig. 1a) and the low pairwise distance between their sequences (0 to 3%) indicated that the first three isolates represent a possible asexual morph of *B. obiones*.

Clones CM6BVc2 and CM6BVc15 were identified as *Decorospora gaudefroyi* despite the high nucleotide divergence between these and *D. gaudefroyi* AF394541 sequences (> 5%); the reasons for this were the high bootstrap value (Fig. 1a) and the fact that this species was previously recorded on the same vegetative structures and study site by morphological methods (Calado et al. 2015).

Some of the clones exclusively detected by molecular methods were only identified to order (CM6BS1c3, CM5LVc4/CM6LS1c12 and CM6LS1c3 representing Pleosporales 1, 2 and 3 respectively) or genus level (CM5LS1c2 representing *Phaeosphaeria*) given the lack of pure isolates and published sequences for comparison at lower taxonomic levels (Table 1, Fig. 1a).

Clones CM6LS1c15 and CM6BS1c4 were identified as *Phaeosphaeria spartinae* based on the close affinity of these clones to BLAST best-hits (Table 1) with which they formed a well-supported clade (Fig. 1a).

Clones CM1LVc8, CM2LVc18, CM3LS2c15 were included in *Stagonospora* clade, which comprised isolates and BLAST best-hits (Fig. 1a). The clone AV3LS1c14 was also representing the genus *Stagonospora* based on the BLAST results (Table 1), but was a different species (Fig. 1a). The positions of *Stagonospora* sp. 1 and 2 in the trees suggested that both species might be asexual morphs of the Pleosporales; *Stagonospora* sp. 1 might also be an asexual morph of *Phaeosphaeria* as this fungus produces seven-septate conidia (Fig. S1 in Calado et al. 2015) as the asexual morphs of *Phaeosphaeria* (Câmara et al. 2002; Kirk et al. 2008; Zhang et al. 2009).

Isolates previously identified as *Phoma* sp.1 (Calado et al. 2015) were found to be the asexual morph of *Phaeosphaeria halima* by BLAST results (Table 1). Clones CM6LS1c2, CM4LVc9, AV1LS1c10, AV3BVc5 were closely related with these isolates and with *P. halima* AF422971 and AF422991 retrieved from GenBank, forming a distinct clade but poorly supported (Fig. 1a); this clade was separated into two relatively well-supported subclades. Pairwise distance between sequences included in distinct subclades varied between 4 and 8%. The high sequence divergence of *ITS* sequences of *P. halima* has already been documented by Buchan et al. (2002), who observed that strains of *P. halima* isolated from *S. alterniflora* and decaying wood grouped in a high-supported clade with a 84% sequence similarity. Clone AV2LVc11, although identified as *P. halima*, was placed in the sister clade representing *Phaeosphaeria spartinicola*, which highlighted the high homology between both species (Fig. 1a). This last clade comprised clones CM1LS1c2, CM2LS1c1, AV1LVc1 and AV3LS2c 7, isolates of *P. spartinicola* and sequences of this species retrieved from GenBank (Table 1, Fig. 1a); nevertheless the low bootstrap value supporting this clade, the pairwise distance between sequences was lower than 2%.

In the maximum parsimony tree of the Sordariomycetes, all the defined OTUs were distinguished (Fig. 1 b).

The *ITS* sequence of the clone AV3CVc6 was compared with several unpublished sequences of the Halosphaeriaceae and showed high homology with *Halosarpheia fibrosa* (pairwise distance between sequences was 5%); considering that *H. fibrosa* has only been described from mangroves, particularly in subtropical regions (Hyde and Lee 1995), and that *Halosarpheia trullifera* was previously recorded in leaf sheaths of *S. maritima* (Calado et al. 2015), it is more likely that the clone represented the latter species. However, the lack of an isolate or published sequences of *H. trullifera* for comparison did not enable an identification to species level (Table 1). Clones AV3BVc7 and CM3BS2c4 also did not cluster with any other taxa or show high affinity with BLAST best-hits (Table 1, Fig. 1b), being designated as unidentified Halosphaeriaceae 1 and 2 respectively given their position on the phylogenetic tree. Clones AV5BVc7 and AV5BS2c2 were identified as *Natantispora retorquens* based on the highly supported clade comprising these clones and isolates of this species (Fig. 1b).

The clone CM6BS1c2 formed a well-supported clade with *Anthostomella brabeji* (EU552098) (Fig. 1b); however, a sequence similarity of 90% to *A. brabeji* (Table 1) and the record of *Anthostomella spissitecta* on the same study site (Calado et al. 2015) suggested that the clone might be best referred as *A. spissitecta* or another species in the same genus.

Maximum parsimony and neighbor-joining trees distinguished an entirely separated and highly supported clade comprising members of Lulworthiaceae (Fig. 1b). Within this clade, clones CM4CS1c3 and CM4CS1c15 grouped in a relatively well-supported subclade, whereas clones AV1BS1c1 and AV5BVc12 and isolates of *Lulworthia* were included in another highly supported subclade; the pairwise distance between sequences of the clones positioned in different subclades was higher than 5%. The higher homology between the sequences of the clones AV1BS1c1 and AV5BVc12 and isolates suggested that all sequences represented the same

species, i.e. *Lulworthia* sp. 1. The higher proximity of this unidentified species of *Lulworthia* to *Lulwoana* (Table 1) than to other species of *Lulworthia* may be related to the polyphyly of the genus (Campbell et al. 2005; Jones 2011b; Pang 2012).

Phylogenetic trees also separated the sequences assigned to Nectriaceae in a well-supported clade, which was, in turn, divided in two subclades representing the genus *Fusarium* and *Cosmospora* (Fig. 1b); clones CM3LVc6 and CM3BS2c1 were identified as *Fusarium oxysporum* and *Cosmospora* sp. respectively, based on the tree and affinity to BLAST best-hits (Table 1, Fig. 1b).

Sequences of the isolates of *Buergenerula spartinae* and sequences retrieved from GenBank formed a monophyletic, highly supported clade with clones AV4BVc2 and CM6BVc13, with a high similarity (> 99%) (Fig. 1b).

In total, the sequences of the 54 representative clones retrieved from the plant samples represented 33 fungal taxa (Table 1). These sequences were then used as a reference database to identify the remaining 842 sequences (436 and 406 retrieved from Castro Marim and Ria de Aveiro samples respectively) (Fig. 2).

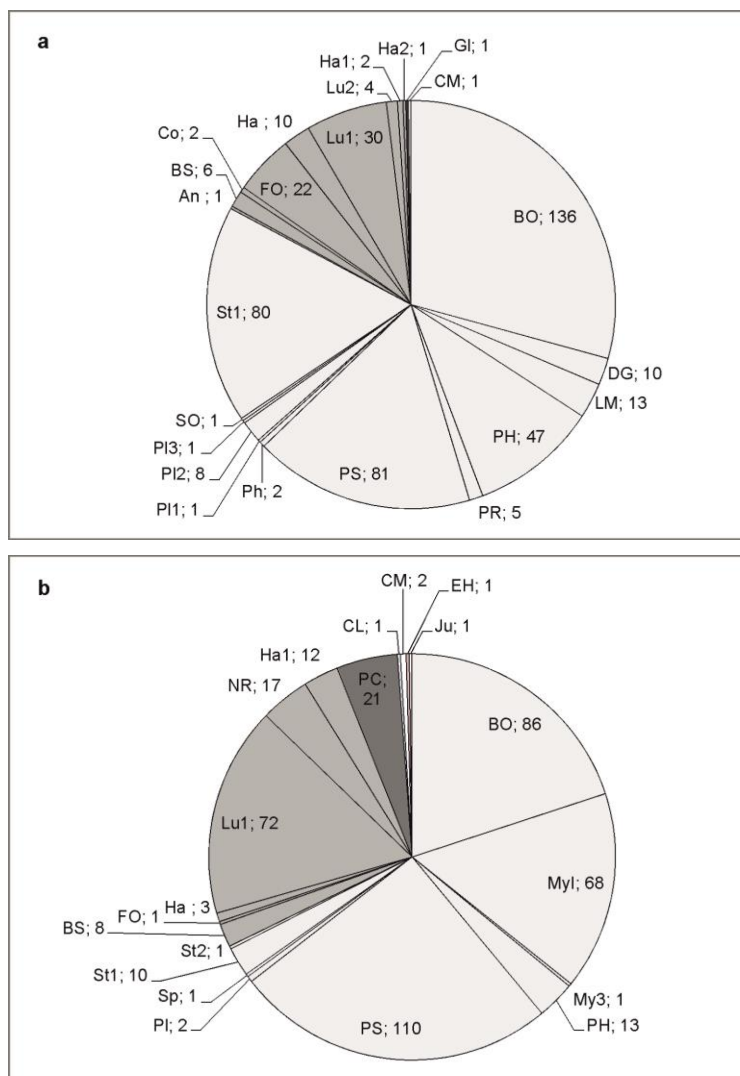


Fig. 2 Number of amplicons assigned to each fungal taxon retrieved from Castro Marim (a) and Ria de Aveiro (b) plant samples: BO *Byssothecium obiones*; DG *Decorospora gaudefroyi*; LM *Leptosphaeria marina*; MyI *Mycosphaerella* sp. I; My3 *Mycosphaerella* sp. 3; PH *Phaeosphaeria halima*; PR *Phaeosphaeria spartinae*; PS *Phaeosphaeria spartinicola*; Ph *Phaeosphaeria* sp.; Pl *Pleospora* sp. ; Pl 1 Pleosporales 1; Pl 2 Pleosporales 2; Pl 3 Pleosporales 3; SO *Sphaerulina orae-maris*; Sp *Sphaerulina* sp.; St 1 *Stagonospora* sp. 1; St 2 *Stagonospora* sp. 2; An *Anthostomella* sp.; BS *Buergenerula spartinae*; CO *Cosmospora* sp.; Fu *Fusarium oxysporum*; Ha *Halosarpheia* sp.; Lu 1 *Lulworthia* sp. 1; Lu 2 *Lulworthia* sp. 2; NR *Natantispora retorquens*; Ha1 Unidentified Halosphaeriaceae 1; Ha2 Unidentified Halosphaeriaceae 2; PC *Penicillium chrysogenum*; Gl *Gloeotinia* sp.; CL *Ceriporia lacerata*; Ju *Junghuhnia* sp.; CM *Cryptococcus mangaliensis*; EH *Erythrobasidium hasegawianum.* Ascomycota and Basidiomycota are represented by grey and white colors respectively, and Dothideomycetes, Sordariomycetes, Eurotiomycetes and Leotiomycetes classes by light, medium, medium-dark and dark gray respectively

The results revealed that *ITS* amplicons more frequently recovered from plant samples corresponded to the most frequently fungi recorded by fruiting structures in the same samples, i.e. *B. obiones*, *L. marina, Lulworthia* sp. 1, *Mycosphaerella* sp. I, *P. halima*, *P. spartinicola* and *Stagonospora* sp. 1 (Fig. 2; Calado et al. 2015). This means that the abundance of amplicons in the clone libraries reflected the real frequencies of occurrence of fungi in Castro Marim and Ria de Aveiro salt marshes. The only exceptions were *N. retorquens* and *S. orae-maris* in Castro Marim plants samples, and *B. spartinae* on samples from both study sites; the first species was not recovered by molecular methods, the second was detected only once, and the third was represented by a very few amplicons (Fig. 2). One of the reasons for these exceptions might be the high melanisation of cell walls of fungal taxa that may interfere in the extraction of DNA (Buchan et al. 2002). The exclusive absence of sequences of *N. retorquens* from Castro Marim samples was totally unexpected and inexplicable.

Even though the identification of the sequences retrieved from plant samples relied mainly on the comparison with published sequences, the sequences of fungal isolates revealed a crucial importance in the identification of the most abundant *ITS* amplicons in the clone libraries (Table 1, Fig. 2). The entire *ITS* regions of the frequent fungal taxa *B. obiones*, *L. marina*, *Lulworthia* sp. 1, *N. retorquens*, *S. orae-maris* and *Stagonospora* sp. 1 were sequenced for the first time in this study. Moreover, the isolation and sequencing of *ITS* rDNA of sexual and asexual morph of *B.obiones* enabled to establish a connection; the asexual morph of *B. obiones* identified in this study might correspond to the one previously described by Kohlmeyer and Kohlmeyer (1979).

Since most of fungal species associated with *Spartina* plants are host-exclusive and temperate fungi (Calado et al. 2015), there is still a lack of fungal *ITS* sequences recovered from species of *Spartina* and salt marsh ecosystems in these databases. Most of the BLAST best-hits were provided by Buchan et al. (2002) study that focused on the mycota inhabiting leaf blades of *S. alterniflora*.

The majority of other sequences and singletons exclusively retrieved by molecular methods were also not represented in public databases and thus were not identified to species level. Exceptions were the ascomycetes *D. gaudefroyi*, *F. oxysporum*, *Penicillium chrysogenum* and *P. spartinae* and the basidiomycetes *Ceriporia lacerata*, *Cryptococcus mangaliensis* and *Erythrobasidium hasegawianum*.

Fungal species richness of communities associated with S. maritima

A total of 45 fungal taxa, mostly Ascomycota, were recorded in *S. maritima* samples; 22 (49%), 12 (27%) and 11 (24%) were identified by molecular, morphological and both methods respectively (Table 1; Calado et al. 2015). The primer sets and cloning procedure enabled the detection of the most frequent fungi recorded by morphological methods (Calado et al. 2015), but also other infrequent, inconspicuous and/or non-sporulating fungal species for which the morphological approach was unable to detect and identify. Basidiomycetes *C. lacerata*, *C. mangaliensis* and *E. hasegawianum*, as well as the ascomycete *P. chrysogenum*, were reported for the first time on *Spartina* substrates. The low representation of single-celled and filamentous basidiomycetes in both salt marshes is consistent with previous studies performed in intertidal (Gessner and Kohlmeyer 1976; Samiaji and Barlocher 1996; Barata 2002; Calado and Barata 2012) or marine ecosystems in general (Kohlmeyer and Kohlmeyer 1979; Jones et al. 2009; Jones and Fell 2012).

The species richness obtained in this study may be slightly overestimated based on the fact that some infrequent fungi identified by morphological and molecular methods might represent the same fungus, such as the following pairs of fungal taxa: *A. spissitecta* and *Anthostomella* sp.; *Fusarium* sp. and *F. oxysporum*; and *H. trullifera* and *Halosarpheia* sp..

Nevertheless, the number of fungal taxa was found to be higher in comparison with other fungal communities inhabiting *S. maritima* (Barata 1997, 2002) or different *Spartina* species (Gessner 1977; Peña and Arambarri 1998; Al-Nasrawi and Hughes 2012), which might be related to the combined methodology used herein.

Similarly as reported by Calado et al. (2015) throughout the observation of fruiting structures, the combination of morphological and molecular methods confirmed that the fungal community inhabiting Castro Marim salt marsh was more species rich than the community of Ria de Aveiro; 34 and 26 fungal taxa were recorded in Castro Marim and Ria de Aveiro plant samples respectively (Fig. 2; Calado et al. 2015). The higher species richness in Castro Marim, which resulted mainly from a high number of less frequent fungi, suggested that this well-preserved study site may offer more suitable biotic and abiotic conditions for fungal colonisation than Ria de Aveiro salt marsh, especially for more sensitive and vulnerable species.

Species-specific ecological preferences

This study focused on a particular phase of *S.maritima* life cycle, which implied different representativeness of the nine plant substrates at the time of collection, in terms of presence and proportion of plant material. This resulted from the time-lag that occurred between the onset of senescence and decay processes in different parts of *Spartina* species. In addition to these differences, substrates were positioned differently along the vertical axis of the standing plants, presenting different micro-environmental conditions in terms of water and salinity contents (Gessner 1977; Kohlmeyer and Kohlmeyer 1979; Barata 2002; Calado et al. 2015).

In this early decay phase, only leaf sheaths under different physiological states were present in all sampling periods and study sites (Table 2), and in similar proportions (data not shown).

Table 2 Number of sampling periods during the first year in which each plant substrate was collected, and absolute and estimated species richness using Chao 2 and Jacknife 2 estimators in each plant substrate, study site (CM Castro Marim; AV Ria de Aveiro) and combination of both study sites

	Live leaf sheaths			Senescent leaf sheaths			Decaying leaf sheaths			Live stems			Senescent stems			Decaying stems			Live leaf blades			Senescent leaf blades			Decaying leaf blades		
	CM	AV	Total	CM	AV	Total	CM	AV	Total	CM	AV	Total	CM	AV	Total	CM	AV	Total	CM	AV	Total	CM	AV	Total	CM	AV	Total
No. Sampling periods	6	6	6	6	6	6	6	6	6	6	6	6	5	4	5	2	_	2	6	6	6	6	6	6	5	6	6
Species Richness	8	13	17	17	7	17	20	8	20	5	5	7	5	4	7	3	_	3	6	6	9	11	6	14	3	6	6
Chao 2	17	28	41	25	8	23	22	13	22	5	5	8	6	9	9	5	_	5	6	7	12	15	7	23	3	7	7
Jacknife 2	18	28	38	28	11	28	26	14	25	7	5	11	9	9	12	5	_	5	8	9	15	19	9	26	3	9	9

Unlike the leaf sheaths that remained attached to the stems as decomposition progressed, most of the lower and decayed leaf blades had already abscised before the collection of plants; this implied that only the uppermost leaf blades, which were mostly alive or senescent, were available at the moment of collection. Most of stems were totally green, even in the lower naked portions, which explained the absence or scarcity of senescent and decaying stems during the sampling time.

The low representativeness of senescent and decaying stems and decaying leaf blades might explain the low species richness observed and estimated by Chao2 and Jacknife 2 estimators for these substrates in comparison with the remaining ones (Table 2); this hypothesis was based mainly on previously studies that documented a high percentage of colonisation, species richness and diversity on drift decaying stems of *S. maritima* (Azevedo et al. 2012) and decaying leaf blades of *S. alterniflora* (Buchan et al. 2002; Walker and Campbell 2010). Similarly, the high species richness observed and estimated for live, senescent and decaying leaf sheaths in both study sites (Table 2) might have resulted from a high availability of these substrates at the time of collection, but may also be related with the morphochemical characteristics of these vegetative structures. Leaf sheaths are wider, with a lower phenolic content, more aerenchyma and structurally tougher but less lignified than leaf blades (Anderson 1974; Graça et al. 2000); stems are more heavily lignified (Hodson et al. 1984) and with a lower nitrogen content than leaves (Cartaxana and Catarino 1997; Curado et al. 2013).

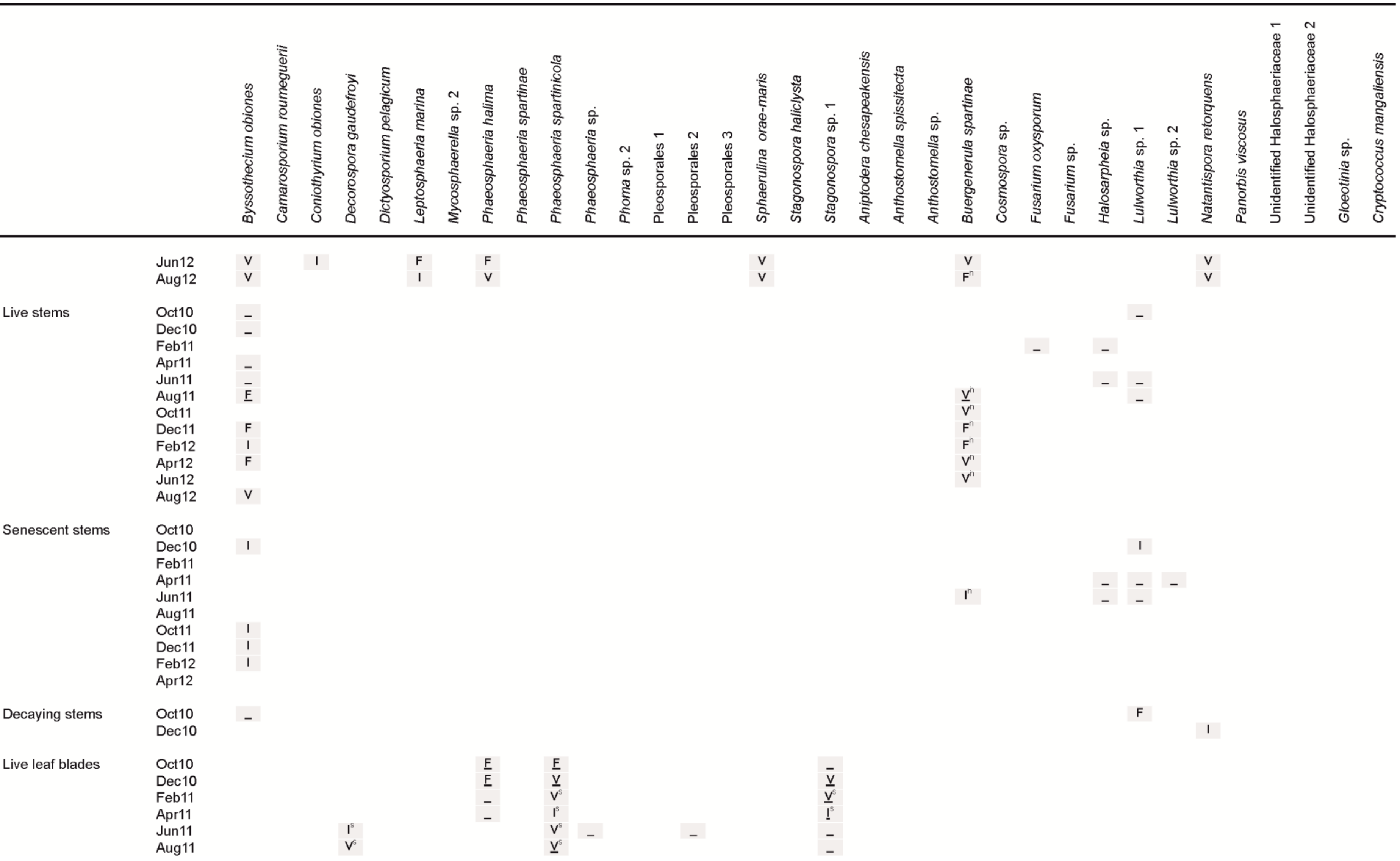
Even though the estimates of species richness pointed out for an insufficient sampling effort (Table 2), the detection of the most frequent fungal taxa by both methods suggested that it was the minimum required to adequately record the most important fungal taxa occurring in this phase of *S. maritima* life cycle.

The comparison of the species composition of communities inhabiting the nine plant substrates revealed that it varied mostly between different vegetative structures (Tables 3 and 4).

Table 3 Percent frequencies of occurrence (V very frequent: >20%; F frequent: 10-20%; I infrequent: <10%, according to the 3 categories proposed by Tan et al (1989)) and/or presences (–) of fungal taxa on different plant substrates in Castro Marim salt marsh in each sampling period, identified by morphological and molecular methods respectively; the superscript letters mark those frequencies of occurrence that were calculated based exclusively on the presence of hyphopodia (^{h}) or spores (^{s})

Leaf sheath	Month	*Byssothecium obiones*	*Camarosporium roumeguerii*	*Coniothyrium obiones*	*Decorospora gaudefroyi*	*Dictyosporium pelagicum*	*Leptosphaeria marina*	*Mycosphaerella* sp. 2	*Phaeosphaeria halima*	*Phaeosphaeria spartinae*	*Phaeosphaeria spartinicola*	*Phaeosphaeria* sp.	*Phoma* sp. 2	Pleosporales 1	Pleosporales 2	Pleosporales 3	*Sphaerulina oraе-maris*	*Stagonospora haliclysta*
Live leaf sheaths	Oct10																	
	Dec10																	
	Feb11																	
	Apr11																	
	Jun11																	
	Aug11	_			_				_									
	Oct11																	
	Dec11																	
	Feb12																	
	Apr12																	
	Jun12																	
	Aug12																	
Senescent leaf sheaths	Oct10	V̲							F̲									
	Dec10	V̲					I		F		I							
	Feb11	V̲					_		F		F̲		I					
	Apr11	V̲	I		_	I	I		V		I							
	Jun11	V̲			_		_										I	
	Aug11	V̲							V	_				_			_	
	Oct11	V		I					V̲		V							
	Dec11	V		I		I			F									
	Feb12	V															I	
	Apr12	V							F									
	Jun12	V							F									
	Aug12	V							F									
Decaying leaf sheaths	Oct10	V̲					V̲		I		I						F	
	Dec10	V̲					V		F		V̲		F				V	
	Feb11	V̲		I			V		V		V		V				V	F
	Apr11	V̲				F	F̲		V̲		V		V				V	
	Jun11	V̲				F	V̲		V								V	
	Aug11	V̲		I			I		V̲				F				V	
	Oct11	V		I		I	V		V		V		I				F	
	Dec11	V		V		I	V		V		V		I				V	
	Feb12	V					V		V		V		I				V	
	Apr12	V					I		V		F						V	I

Leaf sheath	Month	*Stagonospora* sp. 1	*Aniptodera chesapeakensis*	*Anthostomella spissitecta*	*Anthostomella* sp.	*Buergenerula spartinae*	*Cosmospora* sp.	*Fusarium oxysporum*	*Fusarium* sp.	*Halosarpheia* sp.	*Lulworthia* sp. 1	*Lulworthia* sp. 2	*Natantispora retorquens*	*Panorbis viscosus*	Unidentified Halosphaeriaceae 1	Unidentified Halosphaeriaceae 2	*Gloeotinia* sp.	*Cryptococcus mangaliensis*
Live leaf sheaths	Oct10																	
	Dec10																_	
	Feb11							_										
	Apr11																	_
	Jun11					F^{n}												
	Aug11	_				I̲^{n}												
	Oct11					V^{n}												
	Dec11					F^{n}												
	Feb12					I^{n}												
	Apr12					I^{n}												
	Jun12					V^{n}												
	Aug12																	
Senescent leaf sheaths	Oct10										_		V					
	Dec10	I											F					
	Feb11	V							F				F					
	Apr11	I				I^{n}												
	Jun11					V^{n}												
	Aug11				_	V^{n}							I					
	Oct11					V^{n}												
	Dec11					V												
	Feb12	I				V^{n}							I					
	Apr12					V^{n}												
	Jun12					V^{n}												
	Aug12	I				V^{n}												
Decaying leaf sheaths	Oct10		I								V̲		V					
	Dec10										F		V					
	Feb11	F̲					_		F		F̲		V			_		
	Apr11	F											V	I				
	Jun11					F^{n}					F		V		_			
	Aug11			I		V^{n}							V	I	_			
	Oct11					V							V					
	Dec11					V							V					
	Feb12					V							V					
	Apr12					V					I		V					



		Byssothecium obiones	*Camarosporium roumeguerii*	*Coniothyrium obiones*	*Decorospora gaudefroyi*	*Dictyosporium pelagicum*	*Leptosphaeria marina*	*Mycosphaerella* sp. 2	*Phaeosphaeria halima*	*Phaeosphaeria spartinae*	*Phaeosphaeria spartinicola*	*Phaeosphaeria* sp.	*Phoma* sp. 2	Pleosporales 1	Pleosporales 2	Pleosporales 3	*Sphaerulina orae-maris*	*Stagonospora haliclysta*
	Jun12	V		I			F		F								V	
	Aug12	V					I		V								V	
Live stems	Oct10	_																
	Dec10	_																
	Feb11																	
	Apr11	_																
	Jun11	_																
	Aug11	$\underline{F}$																
	Oct11																	
	Dec11	F																
	Feb12	I																
	Apr12	F																
	Jun12																	
	Aug12	V																
Senescent stems	Oct10																	
	Dec10	I																
	Feb11																	
	Apr11																	
	Jun11																	
	Aug11																	
	Oct11	I																
	Dec11	I																
	Feb12	I																
	Apr12																	
Decaying stems	Oct10	_																
	Dec10																	
Live leaf blades	Oct10								$\underline{F}$		$\underline{F}$							
	Dec10								$\underline{F}$		$\underline{V}$							
	Feb11								_		V^{s}							
	Apr11								_		I^{s}							
	Jun11				I^{s}						V^{s}	_			_			
	Aug11				V^{s}						$\underline{V}^{s}$							

		Stagonospora sp. 1	*Aniptodera chesapeakensis*	*Anthostomella spissitecta*	*Anthostomella* sp.	*Buergenerula spartinae*	*Cosmospora* sp.	*Fusarium oxysporum*	*Fusarium* sp.	*Halosarpheia* sp.	*Lulworthia* sp. 1	*Lulworthia* sp. 2	*Natantispora retorquens*	*Panorbis viscosus*	Unidentified Halosphaeriaceae 1	Unidentified Halosphaeriaceae 2	*Gloeotinia* sp.	*Cryptococcus mangaliensis*
	Jun12					V							V					
	Aug12					F^{n}							V					
Live stems	Oct10										_							
	Dec10																	
	Feb11							_		_								
	Apr11																	
	Jun11									_	_							
	Aug11					$\underline{V}^{n}$					_							
	Oct11					V^{n}												
	Dec11					F^{n}												
	Feb12					F^{n}												
	Apr12					V^{n}												
	Jun12					V^{n}												
	Aug12																	
Senescent stems	Oct10																	
	Dec10										I							
	Feb11																	
	Apr11									_	_	_						
	Jun11					I^{n}				_	_							
	Aug11																	
	Oct11																	
	Dec11																	
	Feb12																	
	Apr12																	
Decaying stems	Oct10										F							
	Dec10												I					
Live leaf blades	Oct10	_																
	Dec10	$\underline{V}$																
	Feb11	$\underline{V}^{s}$																
	Apr11	$\underline{I}^{s}$																
	Jun11	_																
	Aug11	_																

Leaf type	Date	*Byssothecium obiones*	*Camarosporium roumeguerii*	*Coniothyrium obiones*	*Decorospora gaudefroyi*	*Dictyosporium pelagicum*	*Leptosphaeria marina*	*Mycosphaerella* sp. 2	*Phaeosphaeria halima*	*Phaeosphaeria spartinae*	*Phaeosphaeria spartinicola*	*Phaeosphaeria* sp.	*Phoma* sp. 2	Pleosporales 1	Pleosporales 2	Pleosporales 3	*Sphaerulina orae-maris*	*Stagonospora haliclysta*	*Stagonospora* sp. 1	*Aniptodera chesapeakensis*	*Anthostomella spissitecta*	*Anthostomella* sp.	*Buergenerula spartinae*	*Cosmospora* sp.	*Fusarium oxysporum*	*Fusarium* sp.	*Halosarpheia* sp.	*Lulworthia* sp. 1	*Lulworthia* sp. 2	*Natantispora retorquens*	*Panorbis viscosus*	Unidentified Halosphaeriaceae 1	Unidentified Halosphaeriaceae 2	*Gloeotinia* sp.	*Cryptococcus mangaliensis*
	Oct11				I[s]						V												I[n]												
	Dec11								F		V												F[n]												
	Feb12				I[s]						V																								
	Apr12										F																								
	Jun12				F[s]						V																								
	Aug12				I[s]						V																								
Senescent leaf blades	Oct10								F		V								F																
	Dec10								F		V								V																
	Feb11								I		V		I						V																
	Apr11								V		F								V																
	Jun11				I[s]				F		V[s]	_							V																
	Aug11			I	V[s]				V	_	V		I		_	_		F	V																
	Oct11			I	I[s]			I	V		V							I	V																
	Dec11										V								V																
	Feb12				F[s]				F		V								V																
	Apr12							I	F		V								V																
	Jun12				V[s]				I		V								I																
	Aug12				I[s]				I		V																								
Decaying leaf blades	Oct10								F		V								I																
	Dec10								I		V								V																
	Feb11								I		F								V																
	Apr11								I		_								I																
	Jun11								F		F								F																
	Feb12										I																								
	Apr12								I		F																								

Table 4 Percent frequencies of occurrence (V very frequent: >20%; F frequent: 10-20%; I infrequent: <10%, according to the 3 categories proposed by Tan et al (1989)) and/or presences (–) of fungal taxa on different plant substrates in Ria de Aveiro salt marsh in each sampling period, identified by morphological and molecular methods respectively; the superscript letters mark those frequencies of occurrence that were calculated based exclusively on the presence of hyphopodia ([h]) or spores ([s])

		Byssothecium obiones	*Camarosporium roumeguerii*	*Dictyosporium pelagicum*	*Leptosphaeria* sp.	*Mycosphaerella* sp. I	*Mycosphaerella* sp. 3	*Phaeosphaeria halima*	*Phaeosphaeria spartinicola*	*Pleospora* sp.	*Sphaerulina orae-maris*	*Sphaerulina* sp.	*Stagonospora* sp. 1	*Stagonospora* sp. 2	*Aniptodera chesapeakensis*	*Buergenerula spartinae*	*Fusarium oxysporum*	*Halosarpheia trullifera*	*Halosarpheia* sp.	*Lulworthia* sp. 1	*Natantispora retorquens*	Unidentified Halosphaeriaceae 1	*Penicillium chrysogenum*	*Ceriporia lacerata*	*Cryptococcus mangaliensis*	*Erythrobasidium hasegawianum*	*Junghuhnia* sp.
Live leaf sheaths	Oct10																								_		
	Dec10																										
	Feb11	_						_	_	_												_		_		_	_
	Apr11															F̲^{n}											
	Jun11																			_	_						
	Aug11	_														I^n							_				
	Oct11																										
	Dec11															I^n											
	Feb12															F^n											
	Apr12																										
	Jun12															I^n											
	Aug12															I^n											
Senescent leaf sheaths	Oct10	_																		_							
	Dec10	V̲																		_	I						
	Feb11	V̲																			I						
	Apr11	V̲														F^n					V̲						
	Jun11	I																		I̲	I̲						
	Aug11	I						_	_				_			F̲^{n}				_	F						
	Oct11	V						I								V^n											
	Dec11	V						I								F^n					F						
	Feb12	V														V^n					I						
	Apr12	V						I	I																		
	Jun12	V														V^n											
	Aug12	F														F^n											
Decaying leaf sheaths	Oct10	F̲																		V	V̲						
	Dec10	V̲																		V̲	V̲						
	Feb11	V̲							I											V	V̲						
	Apr11	V̲		F				I	I											F	V̲						
	Jun11	V̲							I											V̲	V̲						
	Aug11	V̲													I	F				V̲	V̲						
	Oct11	V				I										F^n		I		V	V						
	Dec11	V														I^n				V	V						
	Feb12	V														F^n				V	V						

		Byssothecium obiones	*Camarosporium roumeguerii*	*Dictyosporium pelagicum*	*Leptosphaeria* sp.	*Mycosphaerella* sp. I	*Mycosphaerella* sp. 3	*Phaeosphaeria halima*	*Phaeosphaeria spartinicola*	*Pleospora* sp.	*Sphaerulina orae-maris*	*Sphaerulina* sp.	*Stagonospora* sp. 1	*Stagonospora* sp. 2	*Aniptodera chesapeakensis*	*Buergenerula spartinae*	*Fusarium oxysporum*	*Halosarpheia trullifera*	*Halosarpheia* sp.	*Lulworthia* sp. 1	*Natantispora retorquens*	Unidentified Halosphaeriaceae 1	*Penicillium chrysogenum*	*Ceriporia lacerata*	*Cryptococcus mangaliensis*	*Erythrobasidium hasegawianum*	*Junghuhnia* sp.
	Apr12	V							F											V	V						
	Jun12	V		F				F	F		I					F^{n}				V	V						
	Aug12	V						I	I							F^{n}				V	V						
Live stems	Oct10																										
	Dec10																			_		_					
	Feb11	_																	_	_							
	Apr11																			_							
	Jun11	_																		_		_					
	Aug11	_																	_	_			_				
	Oct11																										
	Dec11																										
	Feb12																										
	Apr12																										
	Jun12															I^{n}											
	Aug12																										
Senescent stems	Oct10																										
	Dec10																										
	Feb11	_																				_					
	Aug11														I					I							
Live leaf blades	Oct10					_			_																		
	Dec10					_		I̲	F				I														
	Feb11					_			_				_				_										
	Apr11					_		I	V				I														
	Jun11					_		I	V̲																		
	Aug11					_		I	V̲														_				
	Oct11							V	V																		
	Dec11							F																			
	Feb12							I																			
	Apr12								V																		
	Jun12							F	V				I														
	Aug12							F	V																		

		Byssothecium obiones	*Camarosporium roumeguerii*	*Dictyosporium pelagicum*	*Leptosphaeria* sp.	*Mycosphaerella* sp. I	*Mycosphaerella* sp. 3	*Phaeosphaeria halima*	*Phaeosphaeria spartinicola*	*Pleospora* sp.	*Sphaerulina orae-maris*	*Sphaerulina* sp.	*Stagonospora* sp. 1	*Stagonospora* sp. 2	*Aniptodera chesapeakensis*	*Buergenerula spartinae*	*Fusarium oxysporum*	*Halosarpheia trullifera*	*Halosarpheia* sp.	*Lulworthia* sp. 1	*Natantispora retorquens*	Unidentified Halosphaeriaceae 1	*Penicillium chrysogenum*	*Ceriporia lacerata*	*Cryptococcus mangaliensis*	*Erythrobasidium hasegawianum*	*Junghuhnia* sp.
Senescent leaf blades	Oct10					V̲		V̲	V̲				F														
	Dec10					V̲		F̲	V̲				V̲														
	Feb11					V̲		F̲	V̲			_	V̲	_													
	Apr11					V̲		V	V̲				V̲														
	Jun11					V̲		F̲	V̲				I̲^{s}														
	Aug11					V̲		V	V̲				F														
	Oct11					V		V	V				V														
	Dec11				I	V		V	V				V														
	Feb12					V		V	V				V														
	Apr12		I			V		V	V				V														
	Jun12					V		V	V				F														
	Aug12					V		F	V				F														
Decaying leaf blades	Oct10					V	_	I̲	V̲	_																	
	Dec10					V		F	V̲				V̲														
	Feb11					F		F	V̲				V														
	Apr11					V̲		V̲	V̲				F														
	Jun11					V		F̲	V̲				I														
	Aug11					V̲		V	V̲				I														
	Oct11					I		F	F				F														
	Dec11					V		I	V				F														
	Feb12					I		V	V				V														
	Apr12					F		I	V				I														
	Jun12							I	F																		
	Aug12					V		V	V				F														

The most frequent fungi found in both salt marshes demonstrated a clear prevalence on leaf sheaths, stems and/or leaf blades regardless their physiological state (Tables 3 and 4). Specifically, *B. obiones*, *Lulworthia* sp. 1 and *N. retorquens* were recorded on leaf sheaths and stems; *P. spartinicola, P. halima* and *Stagonospora* sp. 1 on leaf sheaths and blades; and *B. spartinae* hyphopodia on all vegetative structures.

Mycosphaerella sp. I, an exclusive and very frequent fungus from Ria de Aveiro salt marsh, was found on leaf blades under different physiological states. *L. marina* and *S. orae-maris*, two exclusive and frequent fungi from Castro Marim salt marsh, were only identified on senescent and decaying leaf sheaths.

Even though the remaining species were recorded on particular plant substrates, their infrequency did not allow to accurately clarify their ecological preferences during this phase of plant life cycle.

The comparison of different plant substrates based on the presence/absence of fungal taxa in all the sampling periods of the first year corroborated most of the above-mentioned ecological tendencies (Figs. 3 and 4).

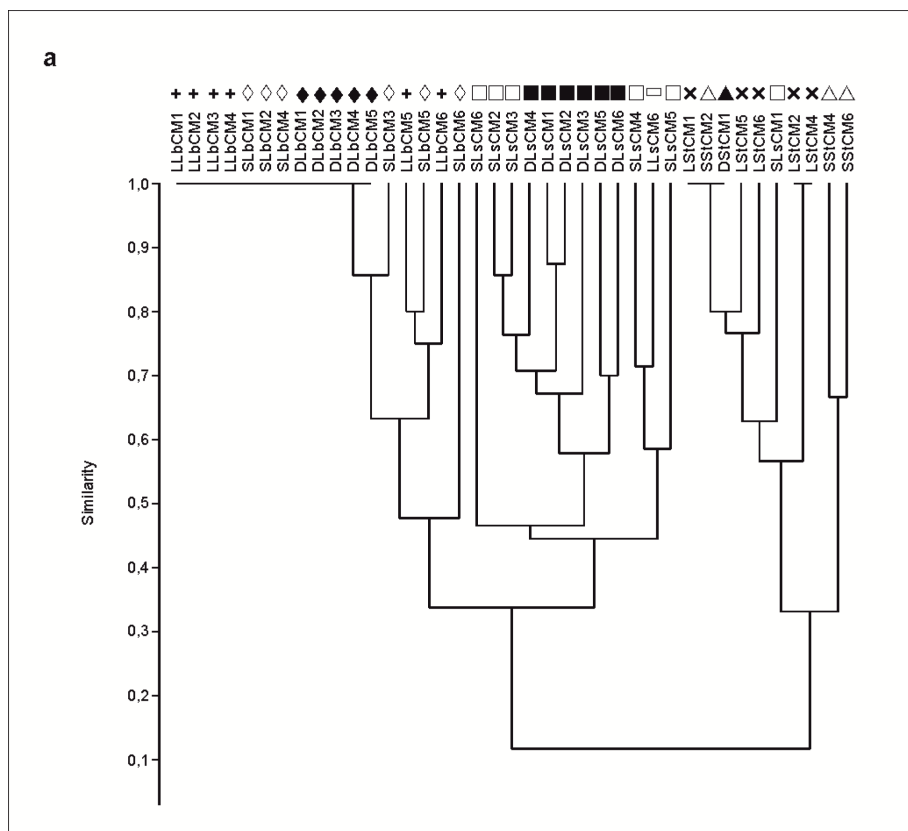


Fig. 3 Cluster dendrogram based on Bray-Curtis similarities of plant substrates, considering the presence/absence of fungal taxa on each plant substrate, in each sampling period and in each study site (a Castro Marim; b Ria de Aveiro). The first three letters of the code name indicate the substrate (LLs-live leaf sheaths, SLs-senescent leaf sheaths, DLs-decaying leaf sheaths, LSt-live stems, SSt-senescent stems, DSt-decaying stems, LLb-live leaf blades, SLb-senescent leaf blades, DLb-decaying leaf blades), the next two letters designate the study site (CM-Castro Marim, AV-Ria de Aveiro) and the number indicates the sampling period (1-Oct10, 2-Dec10, 3-Feb11, 4-Apr11, 5-Jun11, 6-Aug11). Different symbols correspond to different plant substrates: ▫ live leaf sheaths, ☐ senescent leaf sheaths, ■ decaying leaf sheaths, ✕ live stems, △ senescent stems, ▲ decaying stems, + live leaf blades, ◊ senescent leaf blades, ♦ decaying leaf blades

Fig. 4 Nonmetric multidimensional scaling plot based on Bray-Curtis similarities of plant substrates considering the presence/absence of fungal taxa in each plant substrate, in each sampling period and in each study site (a Castro Marim; b Ria de Aveiro). Different symbols correspond to different plant substrates: ▫ live leaf sheaths, ☐ senescent leaf

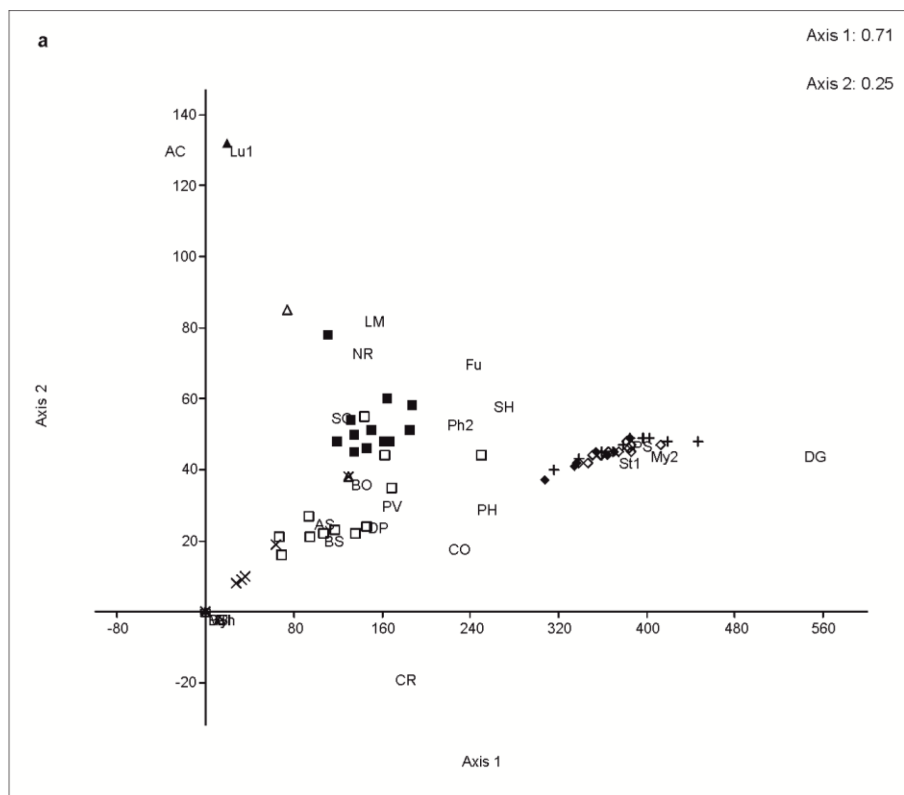
sheaths, ■ decaying leaf sheaths, × live stems, △ senescent stems, ▲ decaying stems, + live leaf blades, ◊ senescent leaf blades, ♦ decaying leaf blades

Cluster analysis and MDS ordination clearly differentiated the samples that represent different vegetative structures, i.e. leaf sheaths, stems and leaf blades, regardless of their physiological states. However, based on the s-stress values provided by MDS analyses (Castro Marim: 0.17; Ria de Aveiro: 0.30), the ordination of Castro Marim samples in the plot represented well the relationships among them, while the Ria de Aveiro samples seem to be more randomly distributed in the 2-dimensional ordination space.

Both analyses revealed a higher similarity between the communities inhabiting leaf sheaths and blades in Castro Marim, and leaf sheaths and stems in Ria de Aveiro salt marsh; stems and leaf blades yielded the most dissimilar fungal communities.

ANOSIM analyses showed significant differences in the fungal communities on plant substrates from both salt marshes ($R=0.7$, $p<0.001$); in the majority of posterior pairwise comparisons, no significant differences were found among substrates representing the same vegetative structure (data not shown).

The comparison of the same plant substrates based on the fruiting patterns of fungal colonisers along a two-year study period by a DCA (Fig. 5) analysis confirmed the existence of three main ecological niches.



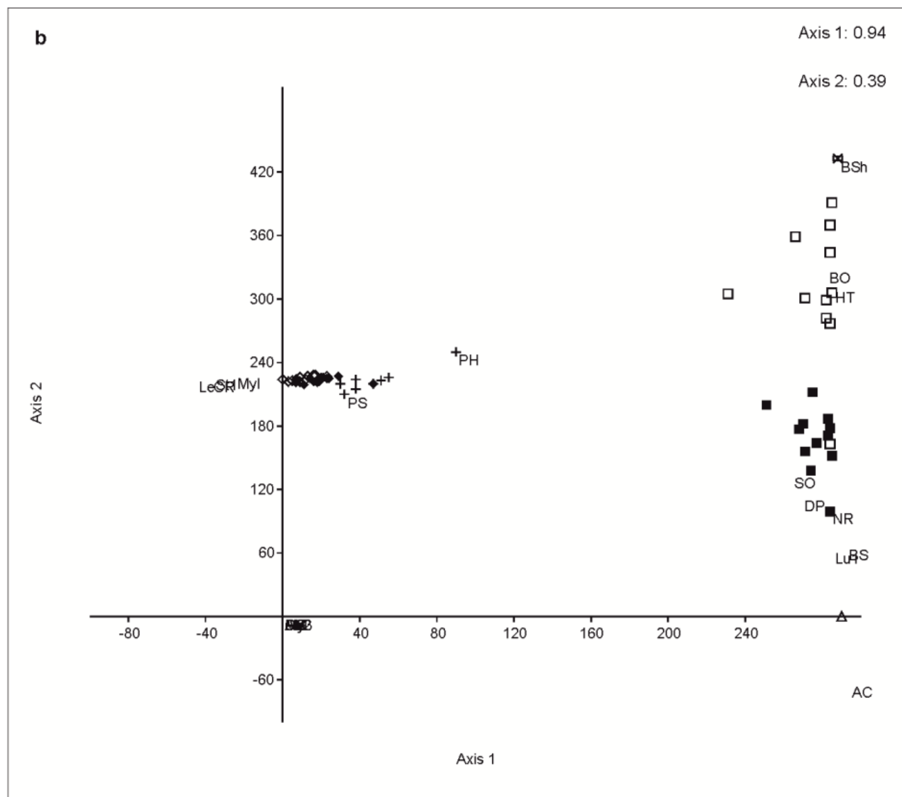


Fig. 5 Two-dimensional DCA plot expressing the spatial distributions of fungal taxa and the nine plant substrates based on the frequencies of occurrence of fungal taxa in each plant substrate, in each sampling period and in each study site (a Castro Marim; b Ria de Aveiro). Different symbols correspond to different plant substrates: ▫ live leaf sheaths, □ senescent leaf sheaths, ■ decaying leaf sheaths, ✕ live stems, △ senescent stems, ▲ decaying stems, + live leaf blades, ◊ senescent leaf blades, ♦ decaying leaf blades. The two-letters code represent fungal taxa: AC *Aniptodera chesapeakensis*, AS *Anthostomella spissitecta*, BO *Byssothecium obiones*, BS *Buergenerula spartinae* ascomata, BSh *Buergenerula spartinae* hyphopodia, CO *Coniothyrium obiones*, CR *Camarosporium roumeguerii*, DG *Decorospora gaudefroyi*, DP *Dictyosporium pelagicum*, Fu *Fusarium* sp., HT *Halosarpheia trullifera*, LM *Leptosphaeria marina*, Lu1 *Lulworthia* sp. 1, MyI *Mycosphaerella* sp. I, My2 *Mycosphaerella* sp. 2, NR *Natantispora retorquens*, PH *Phaeosphaeria halima*, Ph2 *Phoma* sp. 2, PS *Phaeosphaeria spartinicola*, SH *Stagonospora haliclysta*, SO *Sphaerulina orae-maris*, St1 *Stagonospora* sp. 1

The first dimension of DCA-ordination (Fig. 5), with a high eigenvalue for both datasets (Castro Marim: 0.71; Ria de Aveiro: 0.94), clearly separated the samples according to the three vegetative structures represented by them. The higher proximity of the fungal taxa to particular vegetative structures in the plots confirmed the species-specific ecological preferences in this phase of plant life cycle.

Considering the fact that the majority of the most frequent fungal taxa in these communities have been already recorded on other *Spartina* species (Calado et al. 2015), the results from this and similar studies were combined in order to assess the ecological patterns of these fungi.

Byssothecium obiones, *N. retorquens* and *Lulworthia* sp. 1, which colonise preferentially the basal portions of standing *Spartina* plants (Calado et al. 2015), dwell on live, senescent and decaying leaf sheaths and stems naturally positioned (Tables 3 and 4; Newell 1993; Newell et al. 1996a; Barata 1997, 2002; Peña and Arambarri 1998; Cornick et al. 2005) and collapsed

and unrooted stems (Gessner 1977). *Leptosphaeria marina*, a fungus that also occurs on the basal plant portions (Calado et al. 2015), is found inhabiting standing-senescent and -decaying leaf sheaths of *S. maritima* (Table 3) and rooted- and unrooted-decayed stems of *Spartina* spp. (Gessner and Kohlmeyer 1976). *Sphaerulina orae-maris*, a coloniser of middle plant portions (Calado et al. 2015), colonises standing-senescent and -decaying leaf sheaths of *S. maritima* (Table 3; Barata 1997, 2002); this species was also identified on stem baits of the same host plant (Barata 2006). *B. spartinae*, another species that occupies middle plant portions (Calado et al. 2015), develops hyphopodia under certain circumstances, which strongly adhere and penetrate the surfaces of living, senescent and decaying leaves and stems of *Spartina* plants (Tables 3 and 4; Gessner 1977; Kohlmeyer and Kohlmeyer 1979; Barata 1997). Hyphopodia were demonstrated by Onyile and Gessner (1982) to be produced at a wide range of environmental conditions, including less favorable conditions for fungal growth. The presence of hyphopodia may indicate that the ideal conditions for sexual reproduction were not totally achieved. In this study, ascomata of *B. spartinae* were exclusively and infrequently recorded on decaying leaf sheaths (Tables 3 and 4). However, these structures have been previously reported from standing-decaying leaf blades and stems (Gessner 1977; Kohlmeyer and Kohlmeyer 1979; Newell and Wasowski 1995; Newell et al. 1996a, 2000; Barata 1997, 2002; Newell and Porter 2000; Newell 2001a; Buchan et al. 2002; Walker and Campbell 2010) and drift material (Peña and Arambarri 1996; Azevedo et al. 2012) from *S. maritima* and other species of *Spartina*. *Phaeosphaeria halima*, a coloniser of middle plant portions (Calado et al. 2015), inhabits decaying leaf blades of *Spartina* spp., co-occurring with *P. spartinicola* and *Stagonospora* sp. (Tables 3 and 4; Newell and Wasowski 1995; Newell and Porter 2000; Newell and Zakel 2000; Newell 2001a; Buchan et al. 2002, 2003; Lyons et al. 2003; Walker and Campbell 2010). In this study, this species was also recorded on living and senescent leaf blades and on leaf sheaths in all physiological states. However, and contrary to similar studies, this species only differentiated asexual reproductive structures; the factors that trigger the asexual or sexual reproduction in holomorphic species are not yet clearly understood. *Phaeosphaeria spartinicola* and *Stagonospora* sp. 1 extend their distribution area to the top leaf blades, where they are more frequent (Calado et al. 2015) and dominant (Newell and Wasowski 1995; Newell and Porter 2000; Newell and Zakel 2000; Newell 2001a; Buchan et al. 2002; Walker and Campbell 2010). Even though these last mentioned studies had only reported *P. spartinicola* from decaying leaf blades, this species and *Stagonospora* sp. 1 were identified herein on leaves under all physiological states (Tables 3 and 4); on leaf sheaths, they occupy a small area directly adjacent to the ligule. Barata (2006) and Azevedo et al. (2012) also reported *P. spartinicola* and unidentified species of *Stagonospora* from stem baits and drift stems of *S. maritima*, although with a much lower frequency of occurrence. *Mycosphaerella* sp. I, a coloniser of the top plant portions (Calado et al. 2015), occurs exclusively on leaf blades, particularly on the most decayed ones (Table 4; Newell and Wasowski 1995; Barata 1997; Newell 2001a; Walker and Campbell 2010); in this study, this species was also found on live and senescent leaf blades. A positive interaction between *P. spartinicola* and *Mycosphaerella*

sp. II in leaf blades of *S. alterniflora* to suppress competitors (e.g. *P. halima*, *B. spartinae*) has already been suggested by some authors (Newell and Porter 2000; Newell 2001a; Buchan et al. 2003; Walker and Campbell 2010), and might actually have occurred in this study with a different species of *Mycosphaerella*.

Even though the low frequency of the remaining fungal species limited an understanding of their ecological patterns, the combination of data from this and similar studies that focused on fungal communities associated with *Spartina* spp. confirmed or complemented the information about the ecology of species: *A. spissitecta* occurs on decaying leaf sheaths (Table 3; Kohlmeyer and Volkmann-Kohlmeyer 2002); *A. chesapeakensis* colonises senescent leaf sheaths and stems (Tables 3 and 4; Barata 2006); *D. pelagicum* occurs on senescent and decaying leaf sheaths and decaying stems (Tables 3 and 4; Barata 1997, 2002, 2006; Azevedo et al. 2012); *F. oxysporum* is found on all vegetative structures (Tables 3 and 4; Al-Nasrawi and Hughes 2012); *P. spartinae* inhabits senescent and decaying leaves and decaying stems (Table 3; Newell 1993; Newell et al. 1996a; Barata 1997; Peña and Arambarri 1998); *P. viscosus* occurs on decaying leaves and stems (Table 3; Buchan et al. 2002, 2003; Barata 2006; Azevedo et al. 2012).

In conclusion, the results suggested an enrichment of fungi along the decay process of leaf sheaths, stems and leaf blades instead of a real succession of fungal species. Despite the decrease of soluble organic compounds and increase of recalcitrant materials and interspecific competition for space and nutrients as the decomposition progresses, more decayed substrates presented low content of antimicrobial substances. In addition to the enrichment of species, senescent and decaying vegetative structures represented the substrates where the majority of fungal taxa produced fruiting structures (87% and 83% respectively; Tables 3 and 4). This is in agreement with other studies (Newell 1993, 2001a; Newell and Wasowski 1995; Newell and Zakel 2000; Buchan et al. 2002; Cornick et al. 2005). Fungal fruiting structures represent the end result of the decay process and indicate a substantial amount of supportive mycelium within the plant substrate (Newell and Porter, 2000). According to Van Ryckegem and Verbeken (2005), the reduction of carbon compounds and/or changes in the proportions of the different carbon substrates may trigger fungal reproduction.

Moreover, the results revealed that the occurrence of most frequent fungi on different substrates of *Spartina* depends on three main factors: (1) phase of plant life cycle and specifically the availability of each substrate, (2) vertical position of substrates on standing plants and associated micro-environmental conditions (Calado et al. 2015), and (3) potential fungal competitors inhabiting the same substrates. Each of these factors might interfere more or less in the colonisation process of each fungus, depending on its ecological requirements. The majority of these fungal taxa initiate the colonisation process on a particular vegetative structure that might proceed to other vegetative structure throughout the decay process. This fact indicates that dominant fungal taxa exhibited less nutritive restrictions and high physiological versatility in adapting to different ecological niches.

Potential ecological roles

The high occurrence of *B. spartinae*, *B. obiones*, *Lulworthia* sp. 1, *Mycosphaerella* sp. I, *N. retorquens*, *P. halima*, *P. spartinicola* and *Stagonospora* sp. 1 throughout all decay stages of the same vegetative structure (Tables 3 and 4, Fig. 5) strongly indicated an high ecological importance as saprobes. With the exception of *N. retorquens*, all the remaining fungal species were demonstrated to be involved on the decay process of *Spartina* spp. and particularly in the digestion of lignocellulose present in secondary walls and middle-lamellar layer of cell (Torzilli and Andrykovitch 1986; Bergbauer and Newell 1992; Newell et al. 1996b; Newell and Porter 2000; Buchan et al. 2002, 2003; Lyons et al. 2003, 2010). The marine fungi *L. marina* and *S. orae-maris* seem to be also playing an important ecological role as saprobes in the leaf sheaths that have already undergone natural senescence (Table 3).
The detection of *B. spartinae*, *B. obiones*, *Lulworthia* sp. I, *Mycosphaerella* sp. I, *N. retorquens*, *P. halima*, *P. spartinicola* and *Stagonospora* sp. 1 on live plant tissues, either by molecular, morphological or both methods together, raises some questions about the life style of these species before the onset of senescence. It is not possible to unequivocally conclude if these fungi were present as functional or metabolically inactive propagules since the surfaces of plant samples were not-sterilised and the genetic molecule used in this study was not appropriate for selection of active members of community. According to Edgcomb et al. (2011), RNA-based clone libraries are more indicative for identifying active species rather than DNA-based clone libraries. Moreover, the presence of fruiting structures on living tissues did not clearly indicate if the associated mycelial network was inside or outside the plant tissues.
Despite the limitations imposed by the methodology, the host plants did not reveal any visible disease symptoms suggesting that the fungi were not parasites or pathogens. The only exception might be *B. spartinae*, given that hyphopodial-entry mechanism has been associated with a parasitic phase in the species life cycle (Kohlmeyer and Gessner 1976; Kohlmeyer and Kohlmeyer 1979). Hyphopodia may represent part of the strategy of the species to gain competitive advantage in the colonisation of substrate (Kohlmeyer and Kohlmeyer 1979; Barata 1997).
An early endophytic colonisation of healthy plant tissues by the remaining frequent fungi would certainly give them a competitive advantage over pioneer and latter fungal saprobes and explain their dominance during the decay process. This hypothesis was also postulated by Newell (1996) to explain the omnipresence of *P. spartinicola* on standing decaying leaf blades of *Spartina* plants. Even though these frequent species were not mentioned before as marine endophytes of *Spartina* plants (Nagahama 2006; Kandalepas et al. 2015), there is no reason for excluding this hypothesis. The genus *Lulworthia*, *Mycosphaerella*, *Phaeosphaeria* and *Stagonospora* have been demonstrated to include endophytic species associated with intertidal plants (Elsebai et al. 2009; Xing et al. 2011; Sakayaroj et al. 2012; Kandalepas et al. 2015).

Moreover, *B. obiones*, *Mycosphaerella* sp. I, *P. halima* and *P. spartinicola* exhibited a degree of specificity with *Spartina* spp. (Calado et al. 2015), as has been demonstrated for endophytic fungi (Arnold 2007).
For the infrequent fungi, any interpretation about their ecological role would be merely speculative. Moreover, it is also plausible that some of these fungal taxa that have never been documented in marine environments might represent airborne contaminants.

Final remarks

The combination of morphological and molecular methods provided a more accurate representation of the fungal community associated with standing plants of *S. maritima*; 45 fungal taxa were identified on early decaying plants. Moreover, it contributed to a better understanding of community dynamics and ecological patterns and potential roles of the most frequent fungi in an early phase of decay of the host plant. The integration of the results from this study and similar ones that focused on *Spartina* decay system revealed that the presence and ecological role of dominant fungi on different substrates depends on the combination of three main factors: phase of plant life cycle, micro-environmental conditions of substrates and potential fungal competitors.
Further studies should be performed in order to clarify the ecological role of these fungi before the beginning of senescence.

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CHAPTER 4 – Final overview

4.1 General conclusions

The present study contributed to a better understanding of marine fungal communities associated with *Spartina* plants in temperate salt marshes. Specifically, it complemented the inventories of marine mycota in these ecosystems and in Portugal, shedding light on key aspects of the ecology of fungi inhabiting standing-decaying plants of *Spartina maritima*.

As expected, the combination of traditional morphological and molecular identification methods was demonstrated to be advantageous. The results revealed a high agreement between the identification based on direct observation of fruiting structures and on nuclear ITS sequences concerning with assignments to fungal species and representativeness of each fungus in the community, plant portions (basal, middle and top portions), plant substrates (live, senescent and decaying leaf sheaths, stems and leaf blades) and study site (Castro Marim and Ria de Aveiro salt marshes). On the other hand, the use of both methods was crucial for obtaining a more realistic estimate of fungal diversity associated with *S. maritima* plants since it helped overcoming the major drawbacks inherent to morphological and molecular methods.

The fungal communities associated with *S. maritima* and other *Spartina* species over a wide geographic range were demonstrated to be very similar, in terms of species diversity, composition and frequency patterns. These communities are not particularly complex or diverse, being dominated by the same host-genus-exclusive species, i.e. *Buergenerula spartinae*, *Byssothecium obiones*, *Phaeosphaeria halima* and *Phaeosphaeria spartinicola*; these fungal species integrate a very stable core group that, apparently, is not much affected by variations in environmental conditions inherent to different latitudinal positions, different morphochemical structure of other *Spartina* host plants or seasonality. Apart from these frequent host-exclusive fungi, also cosmopolitan fungi *Aniptodera chesapeakensis* and *Dictyosporium pelagicum*, frequently reported from other *Spartina* species, were observed in this study. Even though the frequent fungal taxa *Lulworthia* sp. 1 and *Stagonospora* sp. 1 were not identified to species level, the two genera are usually well-represented in fungal communities associated with *Spartina* plants.

Beyond this similarity, the communities are composed by other fungal species, whose occurrence seems to be determined by other macro and/or micro-environmental factors.

Natantispora retorquens, a cosmopolitan species reported from several tropical ecosystems, was found to be one of the most frequent and dominant species in the fungal communities associated with *S. maritima*, although it has not been reported from other *Spartina* species. Similarly, *Panorbis viscosus*, another cosmopolitan species that has been described from temperate and tropical regions, was infrequently collected in this, Barata (2002) and Azevedo et al. (2012) studies, on plants of *S. maritima*.

Mycosphaerella sp. I, an host-genus-exclusive fungus, very frequent in Ria de Aveiro salt marsh and in other temperate salt marshes, was absent in Castro Marim salt marsh. On the other hand, the temperate non-host-exclusive fungi *Sphaerulina-oraemaris* and *Leptosphaeria marina*

were found to be very frequent in Castro Marim salt marsh but infrequent and absent, respectively, in Ria de Aveiro salt marsh; similarly, *Phaeosphaeria spartinae*, a very common fungi in communities associated with *Spartina* species, was only retrieved by molecular methods from plant samples of Castro Marim salt marsh. *L. marina*, as well as infrequent ascomycetous species *Anthostomella spissitecta*, *Camarosporium roumeguerii*, *Coniothyrium obiones*, *Decorospora gaudefroyi*, *Fusarium oxysporum*, *Halosarpheia trullifera*, *Penicillium chrysogenum* and *Stagonospora haliclysta*, and basidiomycetes *Ceriporia lacerata*, *Cryptococcus mangaliensis* and *Erythrobasidium hasegawianum*, were reported for the first time on *S. maritima* plants. From all these fungal species, only *A. spissitecta*, *F. oxysporum* and *L. marina* have been previously described from other *Spartina* species.

The presence/absence of these host-genus-exclusive or cosmopolitan fungal species in the communities of *S. maritima* may be attributed to differences in the status of conservation, tidal regime and physical configuration of salt marsh ecosystems, intraspecific morphological variations in host plants, or even nutrient or space competition phenomena.

The fungal taxa colonizing standing plants of *S. maritima* were found to exhibit vertical distribution patterns (Fig. 1). Even though the absolute vertical positions of fungal structures produced by common frequent species varied from one salt marsh to another, the relative positions of these structures along the plants were maintained.

Fig. 1 Illustrative representation of vertical distribution of very frequent and frequent fungi on standing plants of *Spartina maritima*: *BO Byssothecium obiones*; *BS Buergenerula spartinae*; *LM Leptosphaeria marina*; *Lu1 Lulworthia* sp. 1; *MyI Mycosphaerella* sp. I; *NR Natantispora retorquens*; *PH Phaeosphaeria halima*; *PS Phaeosphaeria spartinicola*; *SO Sphaerulina orae-maris*; *St1 Stagonospora* sp. 1. Darker areas in the bars represent the locations where the fungi were more commonly observed and the concentrations of fruiting structures were higher. The grey lines represent the extension of the plants occupied by each vegetative structure: *LB* leaf blades; *ST* stems; *LS* leaf sheaths

The molecular identification of fungi from different substrates that were also vertically distributed along the axis of the standing plants, indirectly confirmed the vertical positions of these fungi (Fig. 1).

The more frequently flooded plant portions (basal portions) were colonised by *B. obiones*, *Lulworthia* sp.1 and *N. retorquens*; these species occurred on live, senescent and decaying leaf sheaths and stems.

The middle plant portions were inhabited mainly by *B. spartinae* and *P. halima*; these two species were detected on culms and leaves in all physiological states, respectively. *L. marina* and *S. orae-maris* were also reported on basal and middle plant portions respectively, but occurred only on senescent and decaying leaf sheaths.

The less inundated plant portions (top portions) were colonised by *Mycosphaerella* sp. I, *P. spartinicola* and *Stagonospora* sp.1; *Mycosphaerella* sp. I was found exclusively on leaf blades whereas *P. spartinicola* and *Stagonospora* sp.1 were observed on leaf blades and adjacent areas of leaf sheaths in all physiological states.

B. obiones, *B. spartinae*, *Mycosphaerella* sp. I, *N. retorquens*, *P. halima*, *P. spartinicola* and *Stagonospora* sp. I were vertically distributed over larger areas of standing plants of *S. maritima* (Fig. 1).

Even though the variations in the plants´ heights and proportions of different substrates during decay process and along the axis of standing plants, the vertical distribution range of each fungus seemed to be maintain. The fact that fungal taxa occupied the same relative vertical positions regardless of the phase of plant life cycle, suggests that the distribution range and abundance of these fungi might be determined by the combination of three factors: salinity and flooding conditions, availability of plant substrate and potential fungal competitors inhabiting the same substrate.

The influence of salinity and flooding conditions in the distribution of fungi was clearly revealed by the dominance of the Sordariomycetes, with dissolving unitunicate asci and passive spore-discharge, in the basal portions and of the Dothideomycetes, with bitunicate asci and an active spore-discharge, in the top portions. Distinct morphologies of reproductive structures and mechanisms of spore's dispersal of the fungal taxa distributed along the standing plants reflect different adaptation strategies to marine or terrestrial environments. This finding, which has already been described in some studies (Fell and Newell 1998; Alias and Jones 2000; Barata 2002; Hyde and Sarma 2006), justified the classification of these fungi into obligate and facultative marine fungi. This classification, which followed the same criteria used by Kohlmeyer and his colleagues to distinguish obligate and facultative marine fungi in standing plants of *Juncus roemerianus* (e.g. Kohlmeyer & Kohlmeyer 2001), goes beyond a merely interpretation of the initial definition proposed by the same authors (Kohlmeyer and Kohlmeyer 1979). The criteria used by Kohlmeyers in their classification have been criticized by some authors as being subjective and dependent on personal opinion. Moreover, these authors argued that the tolerance or dependency to seawater submergence cannot be used as a criterion to infer about the origin of fungi; Jones et al. (2009, 2015), one of the critics to Kohlmeyers´ classification, considered all the fungal species recorded in this study as obligate marine fungi. Even though it may be proved that the fungal species designated in this study as facultative marine fungi are not of terrestrial origin but exclusively marine, the information reported herein about the tolerance or dependency on marine conditions remains unchanged since it is factual. Therefore, and while the concepts are not redefined based on more objective and unequivocal criteria and supported by strong ecological evidences, there is no need to adopt a new classification. A

successful redefinition and standardisation of the concepts should rely on a better and more consistent knowledge of the ecology and biology of each fungus.

The results from this study and the comparison with similar ones (e.g. Gessner and Kohlmeyer 1976; Peña and Arambarri 1996; Barata 1997, 2002, 2006; Azevedo et al. 2012) also revealed that the occurrence of fungi on different plant substrates depends on their availability during each phase of plant life cycle. Moreover, the majority of the most frequent species is not strictly associated with a particular vegetative structure, but is capable to colonise different vegetative structures located in a specific vertical position along the decomposition of *Spartina* plants as soon as the structures become exposed and more accessible.

The presence and abundance of fungi in each ecological niche might also depend on their competitive abilities.

Additionally to the species-specific ecological requirements, the results from this study contributed to infer about species-specific ecological roles during early stages of decay of *S. maritima*, particularly of the most frequent fungal taxa in the communities. The presence of *B. obiones*, *B. spartinae*, *L. marina*, *Lulworthia* sp. 1, *Mycosphaerella* sp. I, *N. retorquens*, *P. halima*, *P. spartinicola*, *S. orae-maris* and *Stagonospora* sp. 1 on senescent and decaying vegetative structures in the majority or all the sampling periods indicates a very stable saprobic community; the seasonality only seemed to interfere in number of fruiting structures produced by these fungi.

The majority of these fungi were also recorded on live tissues by morphological and/or molecular methods. The identification of hyphopodia of *B. spartinae* on live stems and leaf sheaths indicated a parasitic phase in the species life cycle. The occurrence of *B. obiones*, *Lulworthia* sp. 1, *Mycosphaerella* sp. I, *N. retorquens*, *P. halima*, *P. spartinicola* and *Stagonospora* sp. 1 on live healthy plant tissues suggested that these fungi might start colonising the plant as endophytes to gain a competitive advantage over the other pioneer fungal saprobes; this strategy may thus explain the high frequency of these fungal taxa later in the decay process.

All of these findings point out for the existence of a very stable and well-adapted fungal community involved in the decay process of *Spartina* plants. Most frequent obligate marine fungi inhabiting lower and middle plant portions play a major key-role in the decomposition of leaf sheaths and stems, whereas facultative marine fungi inhabiting middle and top plant portions assume a major role in the decay of upper stems and leaf sheaths and/or leaf blades.

These fungi exhibit higher ecological plasticity and lesser nutritive requirements than the remaining fungi in the community, being able to explore more efficiently the plant substrates. Among all the frequent fungi, *B. spartinae* and *P. spartinicola* seem to be more active and intensively involved in the decomposition of *Spartina* species, since they were found on all vegetative structures of standing decaying and drift plants of *Spartina* spp..

4.2 Future perspectives

Even though the substantial increase of general knowledge regarding marine fungi that came with the advent and optimisation of molecular techniques, there are still many gaps in this subject. The search for new fungi has been encouraged mainly by the awareness of the potential biotechnological or pharmaceutical applications of secondary metabolites and enzymes produced marine fungi. Therefore, this has been contributing for a change in the main research paradigm, i.e. the purposes of any research project cannot be exclusively scientific or related with management and conservation strategies in order to be funded, but have also to have a direct or indirect applicability to the society and/or industries.

Nevertheless the research motivation, it is crucial to invest more intensively in the assessment of the diversity of the communities on the less surveyed substrates, habitats and/or geographical locations, as well as in the understanding of the dynamics of these communities and ecology and functional role of each fungus in each particular ecosystem, employing microscopy, culture and molecular techniques. All the fungal species recorded in each study should be isolated and preserved in reference collections and/or their genomic sequences, preferentially of barcoding genes, deposited in public repositories, in order to facilitate taxonomic assignments of unknown or misidentified fungi. Moreover, the metabolic profiles and biotechnological potential of all isolates should be explored.

A holistic and integrated knowledge is required to ensure the success of any management strategy in a conservation or exploration and economic profitability perspective.

Moreover, this knowledge will elucidate about the origin, phylogeny and metabolic dependency of fungi on marine conditions, as well as their ecological importance in marine ecosystems, facilitating the redefinition of the terms used to classify the fungi.

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