Universidade de Aveiro Departamento de Biologia
2019

Tânia Filipa Lopes Vicente

Diversity of fungi associated with macroalgae from Ria de Aveiro

Diversidade de fungos associados a macroalgas da Ria de Aveiro

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

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

Dedico este trabalho aos meus pais, pelo incansável apoio e confiança depositados em mim, desde o princípio!

o júri

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palavras-chave

Fungos associados a algas, Filogenia, Sapal, Taxonomia, Diversidade fúngica, *Cladosporium rubrum*, *Hypoxylon aveirense*.

resumo

A Ria de Aveiro tem sido explorada com o intuito de desvendar a comunidade microbiana albergada pelas plantas aqui presentes. As algas, um grupo bem presente, são ainda organismos pouco explorados nesta lagoa. Os fungos são organismos que conseguem estabelecer diferentes relações de simbiose com as algas. Além disso, sabe-se que produzem compostos bioativos com capacidades interessantes como atividade antioxidante, antialgas, antiviral, anti-insectos e antifúngica. Este trabalho teve por objetivo caracterizar a diversidade de fungos associados a diferentes tipos de macroalgas existentes na Ria de Aveiro. A partir de 7 locais escolhidos, aleatoriamente, foram recolhidas 26 amostras de algas pertencentes aos géneros *Ulva*, *Fucus*, *Enteromorpha*, *Gracilaria*, *Cladophora*, *Ceramium*, *Codium* e *Scinaia*. Após isolamento e tipagem molecular, foi feita a identificação de 486 isolados através da sequenciação da região ITS (Internal Transcribed Spacer) pertencente ao cluster ribossomal. Verificou-se que os principais grupos taxonómicos identificados correspondiam aos géneros *Cladosporium*, *Leptobacillium*, "Acremonium-like species", *Alternaria*, *Penicillium*, entre outros ascomicetes. Todos estes géneros (excetuando *Leptobacillium*) foram já referenciados em ambientes marinhos. *Leptobacillium*, um fungo típico de solo, surge neste trabalho como um novo registo presente em comunidades de algas. Neste estudo foi ainda realizada a caracterização detalhada e descrição taxonómica de duas novas espécies: *Cladosporium rubrum* sp. nov. e *Hypoxylon aveirense* sp. nov. A diversidade das comunidades fúngicas de cada amostra foi avaliada recorrendo aos Índices de Shannon, Simpson e Pielou. Estes demonstraram que uma amostra de alga vermelha (não identificada) e duas de *Fucus* revelaram os valores de diversidade mais elevados. Com a finalidade de apurar se existia alguma influência do local de recolha e/ou hospedeiro, recorreu-se a testes paramétricos (ANOVA) e/ou não-paramétricos ("Kruskal-Wallis rank sum"). Apenas foram observadas diferenças significativas no que toca à uniformidade dos isolados pertencentes às comunidades encontradas em algas vermelhas onde este valor é significativamente superior quando comparado com as comunidades das algas verdes. Análises posteriores dos dados obtidos (função *multipatt*) permitiram detetar uma distribuição preferencial de isolados do grupo *Exophiala* pelas algas identificadas como *Ceramium* sp. e *Scinaia* sp., e *Emericellopsis* pelas algas verdes e vermelhas. Em relação aos locais de colheita, verificou-se a associação de alguns géneros fúngicos com determinados locais onde a sua expressão era maior. Estas diferenças poderão estar associadas a fatores abióticos (diferenças de salinidade) ou fatores bióticos (influência antropogénica). Em suma, este estudo contribuiu para o conhecimento da micobiota de algas da Ria de Aveiro, enquanto reforça a necessidade de mais estudos taxonómicos em ambientes marinhos de Portugal.

keywords

Algicolous fungi, Phylogeny, Salt Marsh, Taxonomy, Fungal Diversity, *Cladosporium rubrum*, *Hypoxylon aveirense*.

abstract

Ria de Aveiro has been explored with the aim of unveiling the microbial community sheltered by the plants in this habitat. Algae, a group well established, are organisms little explored in this lagoon. Fungi are organisms which can establish different relations of symbiosis with algae. Beyond this, it is known that these fungi produce bioactive compounds with interesting properties such as antioxidant activity, antialgal, antiviral, antiinsect and antifungal. This study aimed to the characterize the diversity of algicolous fungi associated with different macroalgae in Ria de Aveiro. From the 7 sites randomly selected, it was chosen 26 algae samples belonging to *Ulva*, *Fucus*, *Enteromorpha*, *Gracilaria*, *Cladophora*, *Ceramium*, *Codium* and *Scinaia.* After fungal isolation and molecular typing, it was made the identification of 486 isolates through the sequencing of ITS (Internal Transcribed Spacer) belonging to ribosomal cluster. The main fungal groups detected belonged to the genera *Cladosporium*, *Leptobacillium*, "*Acremonium*-like species", *Alternaria*, *Penicillium* and other ascomycetes. All these (except *Leptobacillium*) were already described in marine environments. *Leptobacillium*, a soil fungus, appears here as a new record in algicolous communities. In addition, this study included the complete characterization and taxonomic description of two new species: *Cladosporium rubrum* sp. nov. and *Hypoxylon aveirense* sp. nov. The diversity of fungial communities of samples was analysed using the Shannon´s, Simpson and Pielou indices. Results demonstrated that a red alga (unidentified) and two samples belonging to genus *Fucus* were the ones with highest diversity. With the aim of investigate if there is some influence of the site sampling and/or host, it was performed parametric tests (ANOVA) and/or non-parametric ("Kruskal-Wallis rank sum") to determine the presence of significant differences between the samples. It was only detected significant differences between the evenness of the samples belonging to red algae, where the evenness is significantly higher than the green algae. Posterior analysis of the data obtained (using the *multipatt* function) allowed the detection of a preference distribution of the isolates belonging to *Exophiala* sp. in samples of *Ceramium* sp. and *Scinaia* sp, and *Emericellopsis* spp. by red and green algae. Considering the sampling sites, it was observed the association of some fungal genera with specific sites where their expression was higher. Such differences between communities can be related with abiotic factors (salinity differences), as well as biotic factors (anthropogenic influence). Concluding, this study contributed for the knowledge about the algicolous fungi communities from Ria de Aveiro, while reinforces the necessity for more taxonomic studies in Portuguese marine environments.

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LIST OF ABBREVIATIONS

"stat" – statistical significance

***actA* –** actin gene

Adonis – Permutational Multivariate Analysis of Variance Using Distance Matrices

ANOVA – Analysis of Variance

BCRC – Bioresource Collection and Research Center

BLAST – Basic Local Alignment Search Tool

CBS – Westerdijk Fungal Biodiversity Institute, German

CGMCC – Centre for Microbiological Culture Collection

***cmdscale* -** Classical (Metric) Multidimensional Scaling

CMG – Collection Micael Gonçalves

CPC – Collection Pedro Crous

CTAB – Cetyl trimethylammonium bromide

dNTPs – deoxy-nucleotide-tri phosphate

EDTA – Ethylenediaminetetraacetic acid

EtBr - Ethidium bromide

***indicspecies* – "**Relationship Between Species and Groups of Sites" package

ITS – Internal Transcribed Spacer region

M – Molar

$MgCl_2$ – Magnesium chloride

ML - Maximum likelihood

MSP-PCR – Microsatellite-primed polymerase chain reaction

MUCL – Mycothèque de l'Université catholique de Louvain

***multipatt* –** Multi-Level Pattern Analysis

MUM – Micoteca da Universidade do Minho

NaCl – Sodium chloride

NH_4OAc – Ammonium acetate

nt – nucleotide(s)

ø – diameter

OA – Oatmeal agar medium

PCoA – Principal Coordinate analysis

PCR – Polymerase chain reaction

PDA – Potato dextrose agar medium

PERMANOVA - Permutational Multivariate Analysis of Variance Using Distance Matrices

***PMCMR* –** Pairwise Multiple Comparisons of Mean Rank Sums

p-value – probability value

rpm – revolutions per minute

SDS – Sodium dodecyl sulfate

SNP - Synthetic nutrient-poor agar

sp./spp. – species (singular)/ species (plural)

TAE – Tris-acetate-EDTA buffer

TE – Tris-EDTA buffer

***tef1-α* –** translation elongation factor 1-α gene

TES – Tris-EDTA-SDS buffer

U - enzyme unit

UPGMA - Unweighted Pair Group Method with Arithmetic Mean

UTHSC – Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA.

UV – ultraviolet radiation

V - Volts

β-tubulin – Beta-tubulin gene

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Introduction

Fungi are organisms with a remarkable versatility which allows them to adapt to different environmental conditions (namely temperature differences), and sometimes to extreme conditions (Klein & Tebbets, 2007; Ogaki *et al*., 2019). So, they have the capacity to inhabit a wide range of environments such as, the aquatic habitats (Urien *et al*., 2019). Beyond this, the fungi can present a huge diversity with recurrent species description (Kavanagh, 2017; Phookamsak *et al*., 2019). In ecosystems they play a key role in organic matter decomposition (Koivusaari *et al*., 2019). One of the most important characteristics of fungi is their ability to degrade diverse compounds due to excreted products like enzymes, which could be useful in industrial applications for human purposes (Duan *et al*., 2019; Venkatachalam *et al*., 2018). There are evidences that the symbiotic relationship established by fungi with other organisms promotes the excretion of products with medical benefits. Such compounds can possess antimicrobial or anti-inflammatory properties (Chen *et al*., 2018; Stierle & Stierle, 2016).

MARINE ENVIRONMENT

The ocean fills most of the Earth surface and comprises about 96.5 % of total water (Shiklomanov, 2009; Singhal & Gupta, 1999), and can reach 97.5 % with the brackish water inclusion (Bärlocher & Boddy, 2016). Despite the big dimension of the ocean, the expected fungal diversity in this environment is lower than in freshwater habitat (Shearer *et al*., 2007), which comprise only 2.53 % of the world hydrosphere (Singhal & Gupta, 1999), and less than the fungal diversity from soils (Azevedo *et al*., 2010). This situation could be explained by the high amount of biomass in coastal systems, which is higher than in open ocean, limiting the fungi distribution to these peripheric habitats where the organic substrata (like seaweeds) is more available for further degradation by their saprophytic activity (Azevedo *et al*., 2010; Kutty & Philip, 2008; Ogaki *et al*., 2019; Ragukhumar, 2017). In fact, it is this fungal property (degradation of organic matter) what makes these organisms so important in the marine environment. This way, fungi play an important role in biogeochemical cycles and food web (Sridhar, 2017). In addition, other factors can affect the spread of fungi. The distribution of determinate marine fungi can be affected by marine agitation, ionic composition and concentration (namely the hydrogen ion activity), hydrostatic pressure and osmotic response, availability of oxygen and substrata for growth, salinity variations (Fan *et al*, 2002; Pang *et al*., 2011), type of vegetation and geographic characteristics (Azevedo *et al*., 2010). There is a possibility that external contaminants such as metals and fungicides may affect the

marine fungi distribution, since this situation has been observed with soil fungal communities (Azevedo *et al*., 2010).

The proximity between sand and sea, the influence of abiotic factors such as wind and rain, and animal colonization could contribute to the transport of fungal spores between the different environments and lead to discovery of the same species in distinct places. The genus *Lulworthia* is a good example of this situation because species belonging to this group have been found in a wide range of marine substrates such as plants and wood (floating or fixed in plants) (Azevedo *et al*., 2017). This versatility makes difficult the definition for a geographic distribution for marine (related) fungi (Garzoli *et al*., 2018). They can be found in all the extension of marine environment, since Antarctic hosts and ice (Ogaki *et al*., 2019) to hydrothermal vents, being present in marine substrata/habitats located at different depths, as well as, in different salinities (Gladfelter *et al.*, 2019).

MARINE FUNGI VS. *MARINE-DERIVED FUNGI*

It is important to understand *what* a marine fungus is and *what* makes it different from other fungal organisms. It is known that the detection of a mycological community collected from a certain marine substrate does not mean that the fungi found are in their optimal conditions of activity or in their ideal niche. Frequently, the fungi isolated on a Petri dish are a result of the presence of spores or some remaining hyphal portion from a non-typical marine fungus "waiting for the suitable conditions to germinate" (Kohlmeyer & Kohlmeyer, 1979). As referred above, the fungal diversity in this habitat is low, even including the microorganisms which are just in a transition environment (facultative marine fungi) (Ragukhumar, 2017). Hereupon we enter in a conflictual theme in mycology: the definition of "marine fungi" (Sarma, 2018).

The first most consensual and ecological definition for marine fungi was suggested by Kohlmeyer & Kohlmeyer, in 1979. These authors introduced the term "obligate marine fungi", to define the fungi capable to "grow and sporulate exclusively in a marine or estuarine habitat". On the other hand, they also labelled the fungal organisms natives "from freshwater or terrestrial milieu able to grow (and possibly also to sporulate) in the marine environment" as "facultative marine fungi" (Kohlmeyer & Kohlmeyer, 1979; Walker *et al*., 2017). However, the definition of marine substrata is unclear which can lead to a misclassification of fungi isolated from the respective substrata. This situation is visible when the mangrove fungi are included in obligate

marine fungi, even occurring in portions of the tree not submerged or in situations where fungi from marine sediment are grouped with terrestrial fungi or marine facultative fungi, being totally unnoticed by marine mycologists (Jones *et al*., 2009; Pang *et al*., 2016).

Currently, for a correct label of marine fungi, it is necessary to classify the substratum where it was collected as marine. Then, the fungi species can be classified as "marine-fungi" if they are "repeatedly recovered from marine habitats". A species which is recurrently isolated from this habitat is a species with the capacity of developing (grow and/or produce fruiting bodies) in marine habitats; have the capacity to create symbiotic relationships with other organisms inhabiting marine ecosystem and is a "species in evolution" or adaptation at genetic/metabolically level (Pang *et al*., 2016). The group of marine fungi "expands" when it is added the "marine-derived fungi" (Sarma, 2018). Marine-derived fungi is a term widely spread, and sometimes can be misunderstood and wrongly defined as fungi isolated from a marine ecological niche. When a fungus is isolated from marine environment or marine substrates, the sporulation process and vegetative growth are unknown. This means that, the fungi found could also survive without the substrate where they were isolated and their origin are unknown (it can also be terrestrial or from freshwater) (Pang *et al*., 2016).

There are more than 1100 fungal species described in marine environment and the marine fungal diversity has been estimated in 10 000 species (Garzoli *et al*., 2018). This number includes obligate, facultative and marine-derived fungi, with distinct diversity levels depending of substrate (Azevedo *et al*., 2012; Ragukhumar, 2017). The main groups of fungi found in marine substrates belong to taxa of Ascomycota, Basidiomycota (Jones & Pang, 2012), Blastocladiomycota and Chytridiomycota (Garzoli *et al*., 2018). Although these organisms can be considered a group of low diversity in marine habitats, the use of improved experimental molecular protocols has shown a larger fungal diversity than it was expected (Azevedo *et al*., 2013), extending from coastal areas to the deep-sea (Corinaldesi *et al*., 2017), including coral reefs (Wainwright *et al*., 2017) and even the Arctic environment (Hassett *el al*., 2016).

Since early, the ascomycetes fungi were dominant among the fungal species found in marine environment, followed by the anamorphic fungi and basidiomycetes (Kohlmeyer & Volkmann-Kohlmeyer, 1991). After almost 30 years later of Kohlmeyer & Volkmann-Kohlmeyer study (1991), the fungal species found triplicated and reached 1112 species described in marine environment (Sarma, 2018). The predominant fungal genera remained in the Ascomycota (mainly

belonging to Sordariomycetes class) and reached a total of 805 species spread by 352 genera. After, it is verified the presence of fungi belonging to Basidiomycota (21 species spread by 17 genera) and other phyla related with Chytridiomycota, which include 26 species (Jones *et al*., 2015; Pang *et al*., 2016). Beyond these filamentous groups, it was possible the detection of 213 unicellular members of ascomycetous yeasts and basidiomycetous yeasts (Sarma, 2018).

FUNGAL ASSOCIATIONS WITH OTHER ORGANISMS AND THEIR IMPORTANCE IN THE "AQUATIC WORLD"

Although the existence of fungi in the ocean has been recognized since the 1850s, their role in the marine ecosystem remained unknown until later, as well as their potential use for human purposes (Ogaki *et al*., 2019; Pang *et al*., 2016; Sparrow, 1936; Zuccaro *et al*., 2008). Currently it is known that fungi are responsible for the degradation of plant and algae matter in marine environment contributing for the biodeterioration of organic substrates in the ocean (Balabanova *et al*., 2018). In addition, fungi can establish interactions with other organisms in the marine environment, which can be beneficial or prejudicial to the hosts. The versatility and plasticity of fungi allow them to establish associations with other organisms in the trophic web, under symbiosis (Hassett *et al*., 2016; Wainwright *et al*., 2017), as parasites (Garzoli *et al*., 2018) or saprotrophic (Raghukumar, 2017). Parasitism is the most common association found between fungi and marine hosts, which favours the fungi development while the host does not receive any rewarding for this relation (Wainwright *et al*., 2017; Ragukhumar, 2017). For example, the association of the parasitic fungus *Aspergillus sydowii* can be prejudicial for their host, the sea fan coral *Gorgonia ventalina* (Yarden, 2014), due to the necrotic lesions on the tissues of the host. This disease is denominated as "Aspergillosis") which can lead to an epidemy affecting these organisms (Burge *et al*., 2013). On the other hand, it arises an example of a beneficial association: the mycophycobiose established between the fungus *Mycosphaerella ascophylli* and the alga *Ascophyllum nodosum* can avoid the desiccation of the host (Garbary & MacDonald, 1995).

Sometimes, some fungi are undetectable by observation in algae external tissues. In fact, the associations between algae and fungi were considered uncommon in the past (Garbary & MacDonald, 1995). Currently, it is known that algae shelter a huge diversity of endophytic fungi (Ragukhumar, 2017). However, the main source of marine-derived fungi in coastal areas are corals (Ogaki *et al*., 2019), representing the marine organism with which fungi mostly establish associations. After corals, algae are the second biggest source of marine fungi. In addition, marine

sponges are also a great source of fungi producers of interesting bioactivities (Henríquez *et al*., 2013).

FUNGI POTENTIAL

Fungi are amazing organisms which can support extreme environmental conditions. A good example is their ability to support a wide range of different temperatures (Ragukhumar, 2017), since the ice in Antarctic (Ogaki *et al*., 2019) to hot deserts (Sterflinger *et al*., 2012). To survive to these stress conditions, filamentous fungi can produce resistant spores or compounds which support their survival in extreme habitats, such as carotenoids, melanin, polyols (Sterflinger *et al*., 2012) and cold-adapted enzymes (Ogaki *et al*., 2019). Another point of interest for the exploration of marine fungi is their capacity to produce such compounds with interesting properties. The associations between fungi and other organisms from marine environments also can promote the synthesis of products with pharmaceutical applications (Oliveira *et al*., 2012).

There is a large range of "ways" for humans to take advantage from the fungi characteristics. The research of bioactive compounds from marine fungi reached a bigger attention when it was isolated products with anticancer and antiviral potential, from a sponge (*Tectitethya crypta*) (Oliveira *et al*., 2012). There are fungal species already described reporting antimicrobial activity against bacteria, against other fungi (mostly against phytopathogenic species) and against viruses. These species belong to genera *Aureobasidium* (Chi *et al*., 2009; Garay *et al*., 2018; Jiang *et al*., 2016), *Colletotrichum* (Lu *et al*., 2000), *Nigrospora* (Carvalho *et al*., 2012; Rukachaisirikul *et al*., 2010), *Leptosphaerulina* (Cui *et al*., 2011), *Arthrinium* (Bloor, 2008; El-Gendy *et al*., 2018; Vijayakumar *et al*., 1996), *Epicoccum* (Baute *et al*., 1978; Madrigal *et al*., 1991; Qian *et al*., 2013) or *Trichoderma* (Calvet *et al*., 1989; Chet *et al*., 1981; Cotxarrera *et al*., 2002; De la Cruz & Llobell, 1999; Jin *et al*., 2017; Leelavathi *et al*., 2014; Liss *et al*., 1985; Oh *et al*., 2002; Yedidia *et al*., 2003; Zhang *et al*., 2015). *Trichoderma*, *Epicoccum* and *Arthrinium* species are also used as biocontrol agents to control infections in plants (Bloor, 2008; Chet *et al*., 1981; Elad *et al*., 1980; Larena *et al*., 2005; Leelavathi *et al*., 2014). In addition, there is a possibility of marine mycota to display anti-allergic activity (Kimura *et al*., 2007), antidiabetic (El-Gendy *et al*., 2018), anticoagulant (Chi *et al*., 2009), antioxidant (Chandra *et al*., 2013; Chi *et al*., 2009; Fernandez-Acero, 2007; Hashem *et al*., 2014; Jiang *et al*., 2016; Saravanakumar *et al*., 2018; Tianpanich *et al*., 2011) or antitumoral properties (Dubourdieu *et al*., 1981; El-Rahman *et al*., 2014; Garay *et al*., 2018; Jiang *et al*., 2016; Oh *et al*., 2002; Pejin & Kamaran, 2017; Pokrovsky *et al*., 2013). It was reported the importance of

marine fungal compounds when it was detected the production of epolactaene by a marine-derived *Penicillium* sp. Such compound is used in the treatment of neurodegenerative diseases (Kakeya *et al.*, 1995).

Also, it is verified great potential come from the algicolous fungi, associated with any macroalgae. These organisms are important sources of enzymes, and they can release essential minerals to the ocean (Ogaki *et al*., 2019). In addition, other reports about algicolous communities have highlighted their antimicrobial, anti-plasmodial (Sridhar, 2017), antioxidant, antialgal, antiviral, antiinsect and selective antifungal abilities, promoted by their bioactive compounds produced (Ogaki *et al*., 2019).

FUNGI RECORDS IN MEDITERRANEAN AND ATLANTIC PORTUGUESE BEACHES

The potential presented by marine fungi has promoted the research aiming at the identification of endophytic fungal communities from seaweeds. In Europe and Mediterranean regions, the marine mycota is well known (Sridhar *et al*., 2012). However, in Portugal the marine fungi remain little explored in coastal areas. The fungal communities assessed are mainly derived from sands of beaches due to the growing concern about public health.

There are mycological studies of Portugal and Mediterranean coasts mainly focusing on sand samples of the beaches. These surveys concluded that in the Mediterranean, the main mycological genera found belonged to *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Mucor*, *Monilia, Cephalosporium*, *Verticillium*, *Chrysosporium* (Azevedo *et al*., 2010), *Acremonium, Fusarium* and *Candida* (WHO, 2003). Some of these fungi can represent a problem for the human health safety, due their pathogenicity to infect human being (WHO, 2003). Then, the microbiological tests made in Portugal were intensified in order to find more pathogenic species in the beaches. Samples recovered from sand resulted in the identification of dermatophytes (possibly with keratinophilic capacity) such as *Trichophyton mentagrophytes*, *T. rubrum* and *Microsporum nanum* among others (Azevedo *et al*., 2010). Also recovered from sand, São José *et al*. (1994) detected fungi belonging to genera *Acremonium*, *Alternaria*, *Arthrobotrys*, *Aspergillus*, *Aureobasidium*, *Beauveria*, *Chrysosporium*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Geotrichum*, *Humicola*, *Nectria*, *Nocardia*, *Oedemium*, *Paecilomyces*, *Penicillium*, *Sporothrix*, *Staphylotrichum*, *Stemphylium*, *Trichosporon*, *Trichothecium* and *Verticillium* (Azevedo *et al*., 2010). *Penicillium* is a group which remains very present in our coast, because it was recently found in sea water,

driftwood and macroalgae (Gonçalves *et al*., 2019[1]). Besides this report, Sridhar *et al*. (2012) analysed the fungal community in seaweed litter in seven beaches of the northwest coast of Portugal and concluded that majority of the species belonged to ascomycetes genera. This fungi group has been reported in different substrata collected in Portugal, such as, wood samples (Sridhar *et al*., 2012), plants (Calado *et al*., 2015) and driftwood in coastal areas (Azevedo *et al*., 2012) and Mira River estuary (Barata, 2006). Basidiomycota is poorly represented, while *anamorphic* fungi seems to be moderately present also in the same samples indicated above (Azevedo *et al*., 2012; Sridhar *et al*., 2012).

Despite these surveys, the fungi communities associated with algae in Portugal remain little explored (Gladfelter *et al.*, 2019). So, it is necessary the development of studies aiming at characterising the mycobiotaof seaweeds, due the potential presented by these fungal communities.

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Chapter I

Fungi Diversity in Algae from Ria de Aveiro

1. INTRODUCTION

1.1. ALGAE ROLE IN MARINE ECOSYSTEMS

The geographic distribution of algae is predominantly located in coastal areas, with emphasis on macroalgae communities. These organisms represent the second largest source of marine-derived fungi, next to corals, and are crucial to maintain the stability of coastal ecosystems due to their ability to promote nutrient cycling. Macroalgae can be classified, based on their photosynthetic pigmentation: green algae, brown algae and red algae, the most important groups of organization (Pereira, 2009). Within the macroalgae groups, the green algae are widely distributed in littoral area (intertidal zone, where the sun exposition is better), while red and brown algae are easily found in subtidal zone (area with restricted sunlight) (Ogaki *et al*., 2019).

"Algicolous fungi" refers to the fungal species associated with algae hosts. In the last years, these communities have gained more attention due to the potential of their bioactive compounds (Ogaki *et al*., 2019; Raghukumar, 2012; Raghukumar, 2017). These fungal assemblages can act like endophytes (mainly parasites), saprobes, mycophycobionts and/or lichenicolous fungi in the hosts tissues adapted to marine habitats (Sridhar, 2017; Raghukumar,2012).

1.2. FUNGI ASSOCIATION WITH ALGAE

Early on, it was already understood that microscopic fungi were abundant in algae tissues (Cotton, 1907) giving origin to algicolous fungi communities, previously, considered parasites or symbionts in the host tissues (Kohlmeyer & Volkmann-Kohlmeyer, 2003*;* Ogaki *et al*., 2019). There are records of fungi associated with algae described in 1869 (Nave, 1869), and in the 1900, studies regarding marine fungi were intensified (Cotton, 1907; Sparrow, 1936; Sutherland, 1916).

Microscopic fungi are an important component of fungal assemblage in macroalgae (Kohlmeyer & Volkmann-Kohlmeyer, 2003*;* Ogaki *et al*., 2019). However, their expression in the external tissues of the host is not always visible in situations of asymptomatic associations between algae and fungi (Jones *et al*., 2008; Raghukumar, 2012). Thus, it can difficult the fungal detection (Raghukumar, 2012) due to the lack of visible evidences of fungi presence in external algae tissues (Jones *et al*., 2008). The introduction of molecular techniques and improvement of protocols allowed the detection and description of the filamentous fungi in the alga genus *Fucus*, specifically localized in algae thalli. Using the technique LSU rRNA PCR-DGGE (Polymerase Chain Reaction-

Denaturing Gradient Gel Electrophoresis), the researchers mostly found species belonging to Halosphaeriaceae, Lulworthiaceae, Hypocreales, and Dothideomycetes groups (Raghukumar, 2012).

In addition, the importance of microbiota (where are included the fungi) is also related with the benefits conferred to the algae host (Loos *et al*., 2019), as the nitrogen capture promoted by the fungi association (Du *et al*., 2019). Another example of an advantageous association between the host and the endophyte is the relation established between the alga *Fucus serratia* and *Corollospora marina* (previously identified as *Sigmoidea marina*). *C. marina* seems to have an apparent nutritional dependence from the host, because it was only found associated with *Fucus serratia* thalli, when this organism was alive. On the other hand, this association confers to the alga, protection against desiccation (Zuccaro *et al*., 2008).

1.3. ENDOSYMBIOTIC FUNGAL COMMUNITIES

It is unknown *what* exactly promotes differences in endosymbiont fungal communities between different algae and/or environments. It has been suggested that this fungi distribution can be affected by abiotic and/or biotic conditions and does not depend only on the host (Calado *et al*., 2015; Raghukumar, 2012). In 2010, it was suggested that the geographic factor was determinant in the endosymbiont assemblage, independently from the algae host where it was recovered (Harvey & Goff, 2010; Raghukumar, 2012). In 2015, Calado *et al*., reinforced the importance of abiotic factors in determining the fungal community in *Spartina maritima*, where the researchers defend a scenario of species-specific ecological patterns. In addition, this study, carried out in Ria de Aveiro, concluded that abiotic conditions could affect the origin of fungi, as well as their physiological and morphological adaptation (Calado *et al*., 2015). Salinity, air exposure and water submersion were considered the minor influencers in changes verified in fungal community of *S. maritima*, while substrate availability was proposed as the main determinant factor in fungi plant arrangement (Calado *et al*., 2015; Sridhar, 2017).

As happen in peatlands (Artz *et al*., 2007), the fungal communities in marine environment and/or associated with seaweeds also undergo changes promoted by the climate phenomena (Du *et al*., 2019; Loos *et al*., 2019). The distribution of fungal communities as well as their changes and analysis of richness patterns and species dominance, could be important to realize the consequences of climate changes in Antarctic Peninsula marine biota, where the temperature

increases faster. Ogaki (2019) explain this situation based on the proportions between endemic (fungi capable of grow and reproduce only in Antarctica, also denominated "true psychrophilic"), indigenous (species "substrata-specific") and cosmopolitan fungal species (microorganisms capable of grow and reproduce under different abiotic conditions and inhabit a wide range of ecological niches). The climate changes in Antarctic can provoke an increase of cosmopolitan species in algicolous fungi and this way exhibit a scenario resulting from these climate changes (Ogaki *et al*., 2019), allowing the prediction of futures consequences came from global warming.

1.4. MARINE-DERIVED FUNGI IN PORTUGUESE COAST AND AVEIRO

Ria de Aveiro is a salt marsh estuarine environment near the coast on the northwest of Portugal and connects to the sea through one single transition channel (Pereira *et al*., 2009). This lagoon is permanently in contact with the Atlantic Ocean.

Calado *et al*. (2015) studied the filamentous marine fungi associated with the decomposition of *S. maritima* in Ria de Aveiro (salt marsh). In addition to salinity level, this study detected the "substrate factor", as an important parameter to define the fungal community on dead vegetal matter in saline environment. Despite the salinity preferences verified for the growth of some species in this experiment, all the species tested in culture media with different sodium chloride concentration exhibited a great capacity of adaptation, even in low salinity concentration. The main fungal community found in this survey belong to Ascomycota group, highlighting species belonging to the Pleosporales, Microascales and Capnodiales orders (Calado *et al*., 2015). Once again, the active role played in the decomposition of this plant suggest a great importance of the fungi community in algal biodeterioration in marine environments as well as, exhibits a fungal arrangement well adapted to the habitat (Calado *et al*., 2015).

The mycobiota belonging to plants of Ria de Aveiro also was investigated (Aleixo, 2013). In a study performed in 2013 in the Ria de Aveiro, which aimed at characterising the fungal endophytic community of a halophyte, *Halimione portulacoides*, it was detected mostly fungi of orders Pleosporales and Hypocreales (Aleixo, 2013). In less abundancy, it was also identified fungi belonging to orders Xylariales, Botryosphaeriales, Trichosphaeriales, Eurotiales and Pezizales distributed by three different sites in a central area of the Ria de Aveiro. For the first time, this study reported a high diversity and abundance of *Neocamarosporium* associated with this plant in Ria de Aveiro, a genus belonging to Pleosporales and well adapted to saline environment (from saline

water to hypersaline soils) (Aleixo, 2013). In addition, *Neocamarosporium* sp. was also recently detected in a dead tissue of the seagrass *Zostera noltii* (from Ria de Aveiro) and saline water of a south beach (Vila Real de Santo António) (Gonçalves *et al*., 2019[2]).

2. OBJECTIVE

The mycobiota of plants collected from Ria de Aveiro has been analysed over the years (Aleixo, 2013; Calado *et al*., 2015). However, an interesting substrate still unexplored are the macroalgae present in this lagoon. Algal-derived fungi can produce bioactive compounds with interesting activities beneficial for the ecosystems where they are inserted (antifungal and antialgal activities) or useful for the human (antibiotic and antioxidant activities) (Ogaki *et al*., 2019). Although the importance of algicolous fungi is "settled", the diversity of these communities is little explored. This way, this study aimed to explore the mycobiota of algae collected from Ria de Aveiro, an estuarine and coastal lagoon.

The aim of this study was to assess the fungal community of algae from Ria de Aveiro, using a culture-dependent approach.

3. MATERIAL & METHODS

3.1. CULTURE MEDIA

Two culture media were used throughout the work and their compositions are given below. The quantities of the components of each medium are given for a final volume of 1L. After hydration, all media were sterilized by autoclaving at 121 ^{o}C for 20 minutes.

3.1.1. ALGICOLOUS FUNGI ISOLATION

PDA ("Potato Dextrose Agar"), with sea salts and antibiotics addition

TABLE 1 | Composition of the medium used for algae sampling. Quantities necessary for a final volume of 1 L.

Potato Dextrose agar, (Merck)	
Potato Dextrose Agar	39 g
Sea salts	30 g
Tetracycline	0.1 g
Streptomycin	0.1 g

PDA ("Potato Dextrose Agar"), with sea salts

TABLE 2 | Composition of the medium used for fungi cultivation. Quantities necessary for a final volume of 1 L.

Potato Dextrose agar, (Merck)	
Potato Dextrose Agar	39 g
Sea salts	30

3.2. SAMPLE COLLECTION

Algae were collected (manually), from randomly selected sites in the Ria de Aveiro (Figure 1). All the locations of samples collection were recorded, and all the algae were individually stored (4 ^{o}C) in sterilized 50 mL falcon tubes and identified. A total of 26 algae samples were obtained from 7 different sites. To facilitate the algae identification, the sampling site was identified with a letter and each alga was identified with a different number (Table 3).

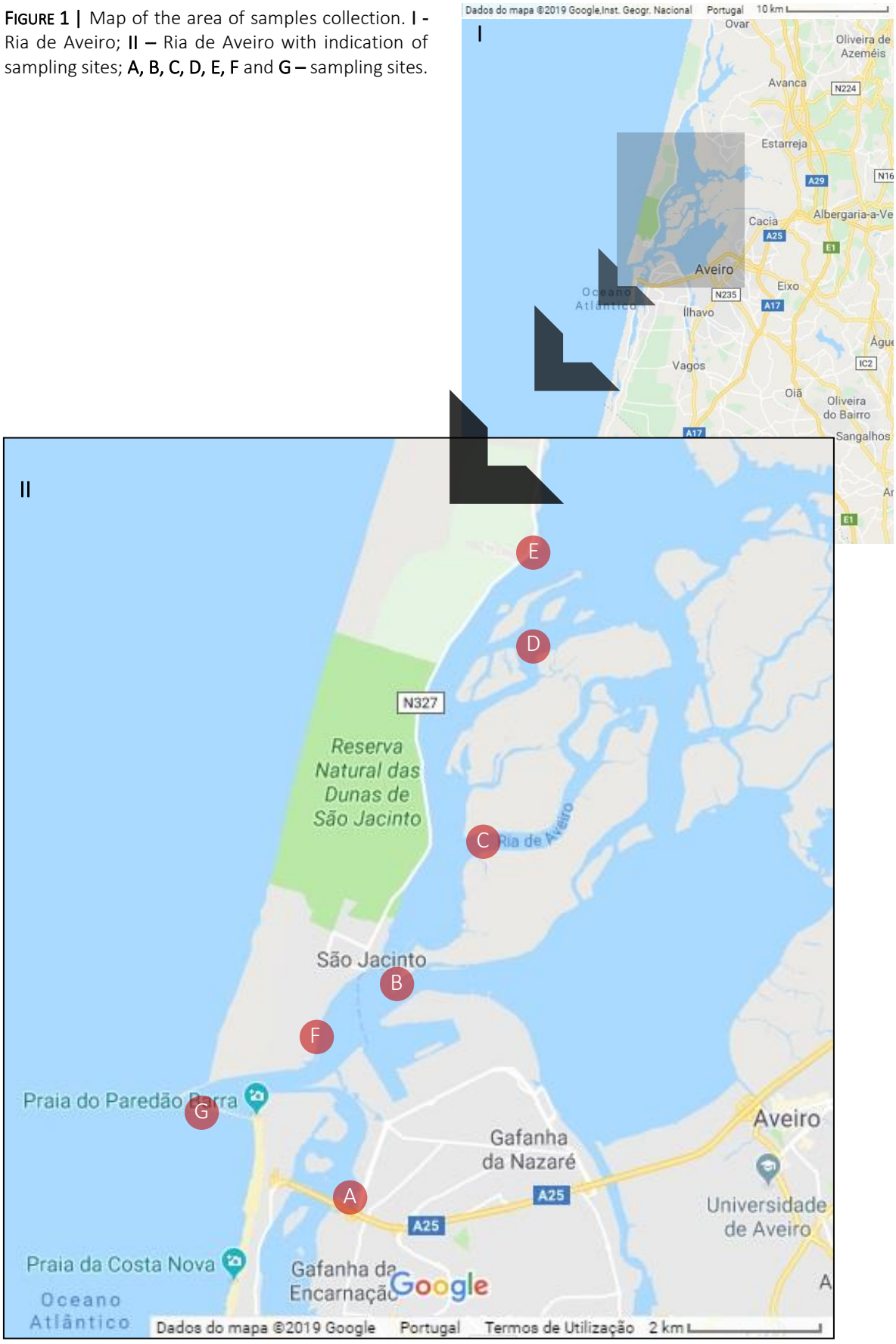

FIGURE 1 | Map of the area of samples collection. **I -** Ria de Aveiro; **II –** Ria de Aveiro with indication of sampling sites; **A, B, C, D, E, F** and **G –** sampling sites.

TABLE 3 | Identification of sampling sites and algae. The two left columns identify the sampling site (letters and coordinates of the site) and the two right columns identify the algae samples (each number correspond to each alga sample). Red alga 1 – algae samples belonging to the same genus. *- unknown algae genus.

Sample collection identification		Algae	
Sampling site	**Site coordinates**	**Algae Identification**	**Genus identification**
A	40.629352, -8.724269	1	*Ulva* sp.
A	40.629352, -8.724269	2	Red alga 1*
A	40.629352, -8.724269	3	*Ulva* sp.
A	40.629352, -8.724269	4	Red alga 1*
A	40.629352, -8.724269	5	*Fucus* sp.
A	40.629352, -8.724269	6	*Fucus* sp.
B	40.659237, -8.724269	7	*Fucus spiralis*
B	40.659237, -8.724269	8	*Fucus spiralis*
B	40.659237, -8.724269	9	*Enteromorpha* sp.
B	40.659237, -8.724269	10	*Fucus* sp.
B	40.659237, -8.724269	11	*Fucus* sp.
B	40.659237, -8.724269	12	*Fucus* sp.
C	40.677377, -8.705795	13	Filamentous green algae*
C	40.677377, -8.705795	14	*Fucus* sp.
C	40.677377, -8.705795	15	*Gracilaria gracilis*
C	40.677377, -8.705795	16	*Ulva* sp.
C	40.677377, -8.705795	17	*Ulva* sp.
D	40.709662, -8.699279	18	*Cladophora* sp.
D	40.709662, -8.699279	19	*Cladophora* sp.
E	40.716893, -8.701211	20	*Ceramium* sp.
E	40.716893, -8.701211	21	Red algae*
E	40.716893, -8.701211	22	*Ulva* sp.
E	40.716893, -8.701211	23	*Enteromorpha intestinalis*
F	40.648426, -8.739905	24	*Fucus* sp.
G	40.641766, -8.757844	25	*Codium* sp.
G	40.641766, -8.757844	26	*Scinaia* sp.

3.3. FUNGAL ISOLATION

Plates with PDA medium, including 3 % sea salts and antibiotics [streptomycin and tetracycline at 100 mg/L (final concentration)], were prepared and stored at 4 ^{o}C until algae processing. For this medium, antibiotics were added to prevent bacterial contaminations. In the laboratory, sample processing was performed under aseptic conditions. First, the algae were

identified at genus level (whenever possible) and washed with sterile sea water. Then, all the algae samples were cut into small pieces with a scalpel and placed on the petri dish containing the medium. Twenty small pieces of each alga were cut and distributed by two Petri dishes. The culture media plates were sealed with Parafilm® and incubated for five days at 25 ºC, to promote fungal growth.

After 48h, 62h and 86h, mycelia growing from algae tissues were transferred to a novel Petri dish containing PDA and incubated at 25 ºC. The process was repeated, in sterile conditions, until pure cultures were obtained.

3.4. FUNGI CONSERVATION

After obtaining growth of the filamentous fungi on PDA supplemented with sea salts, a "cutting disc" was used to obtain four plugs (5 mm ø) for each isolate which were then stored in 1 mL of sterilized glycerol (15 %), and kept at room temperature overnight before being transfer for -80 ºC. All plugs were taken from the margins of the cultures.

3.5. FUNGAL ISOLATES IDENTIFICATION

3.5.1. DNA EXTRACTION

After 7-30 days of incubation in PDA supplemented with sea salts at 20ºC or 25ºC, the total genomic DNA was extracted from fresh mycelium scrapped from the petri dish, according to Möller *et al*., 1992 (see supplementary information S1). In order to perform the DNA extraction, several stock solutions (Table 4, 5 and 6) were prepared and sterilized by autoclaving at 121 ºC, for 20 min.

Solutions for DNA extraction procedure

TABLE 4 | Composition of TES buffer for a final volume of 100 mL.

TES buffer (100 ml)	
1 M Tris (pH = 8)	10.0 mL
0.5 M EDTA (pH = 8)	2 mL
10 % SDS	20 mL

TABLE 5 | Composition of TE buffer for a final volume of 100 mL.

TE Buffer (100 mL)	
1 M Tris (pH = 8)	1 mL
0.5 EDTA (pH = 8)	0.2 mL

TABLE 6 | Solutions used in DNA extraction procedure.

Other solutions (concentrations)
20 mg/ml Proteinase K
5 M NaCl
10 % CTAB
Chloroform: Isoamyalcohol (24:1)
5 M NH_4OAc
76 % Ethanol

3.6. MOLECULAR TYPING

3.6.1. PCR FINGERPRINTING

A genome analysis, using genetic markers, was carried out to discriminate between fungal species (Alves *et al*., 2007). For this purpose, a microsatellite-primed polymerase chain reaction (MSP-PCR) was performed in order to obtain DNA fingerprints, using a set of primers specific for the simple repetitive DNA $(GTG)_5$ described in Table 8 (Alves *et al*., 2007; Meyer *et al*., 1993; Meyer *et al*., 1997).

TABLE 7 | Solution for MSP-PCR using the $(GTG)_5$ primer. All the components were included in all PCR reactions except for negative control, where the DNA was changed by sterilized water.

Microsatellite-primed polymerase chain reaction (MSP-PCR)	
NZYTaq 2× Green Master Mix	6.25 µL
$(GTG)_5$ primer	2 µL (10 pmol/µL)
DNA template	1 µL
Sterile Mili-Q water	15.75 µL

For each PCR reaction, the following reagents were used: NZYTaq 2× Green Master Mix, sterile Milli-Q water, $(GTG)_5$ primer and DNA template (in a dilution of 1:10), making up a total volume of 25 µL (Table 7). PCR reactions started by an initial denaturation at 95 ^oC for 5 minutes, followed by 30 cycles of denaturation at 94 ^oC for 1 minute, annealing at 53 ^oC during 1 minute and extension at 65 ^oC for 8 minutes, concluding with an elongation step at 72 ^oC, at 10 minutes (Alves *et al*., 2007). The Bio-Rad C1000 touch^{TM} Thermal Cycler (USA) was used.

TABLE 8 | PCR conditions adopted for MSP-PCR amplification.

Microsatellite-primed polymerase chain reaction conditions				
Primer	Sequence (5´→ 3´)	PCR conditions		
		Denaturation	Annealing	Extension
$(GTG)_5$	GTGGTGGTGGTGGTG	94 °C, 1 min	50 °C, 1 min	72 °C, 2 min

After microsatellite-primed PCR, a 1.5 % agarose gel was prepared and loaded with 5 µL of each sample. A DNA ladder was loaded on the first and last well of each gel (GeneRuler DNA Ladder Mix 0.5 µg/µL, 50 µg, Thermo Scientific ™, USA). Electrophoresis was performed at 80 V, during 5 h in TAE 1× buffer (40 mM Tris, pH 7.6; 20 mM acetic acid; 1 mM EDTA). The gel was stained with a solution of ethidium bromide (5 µl/mg) for 20-30 minutes, washed in distilled water for 20-30 minutes and image captured under UV light using a GELDOC XR+ system (Bio-Rad, USA).

All fingerprint profiles were stored for further analysis with the GelCompar II software (Applied Maths, Belgium). The Pearson correlation coefficient was applied to assess the relationship between the genetic profiles. In addition, the unweighted pair group method with arithmetic mean (UPGMA) algorithm was used for cluster analysis. This analysis produced a dendrogram which was carefully examined in order to obtain groups of isolates with at least 80 % similarity. All the representative isolates were randomly selected for further identification. This cut-off resulted in 213 isolates selected.

3.7. DNA SEQUENCING

In order to identify the fungal isolates, the Internal Transcribed Spacer (ITS) region of the rDNA cluster was amplified by PCR using the set of primers ITS5 and NL4 (Table 9).

All PCR reactions were prepared for a final volume of 25 µL, including NZYTaq 2× Green Master Mix, sterile Milli-Q water, primers combinations (ITS5 and NL4) and DNA template (diluted 1:10). All components were applied according the proportions stated in the Table 10. For negative control, the components of the reaction were the same, but 1 µL of Milli-Q water was added instead of DNA template.

PCR reactions started with an initial denaturation at 95 °C, for 5 minutes, followed by the conditions indicated in Table 10, and ended with a final elongation of 10 minutes, at 72 °C (Table 11). The Bio-Rad C1000 touch™ Thermal Cycler (USA) was used for ITS amplification.

TABLE 9 | Nucleotide sequences of the primers used for ITS amplification.

Primers DNA sequences used		
Locus	**Primer**	**DNA sequence (5´→ 3´)**
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG
	NL4	GGTCCGTGTTTCAAGACGG

TABLE 10 | PCR composition adopted for each tube used in ITS amplification. All the components were included in all PCR reactions except for negative control, where the DNA was changed by sterilized water.

PCR composition for ITS amplification	
NZYTaq 2× Green Master Mix	6.25 µL
Forward primer (ITS5)	1 µL (10 pmol/µL)
Reverse primer (NL4)	1 µL (10 pmol/µL)
DNA template (diluted 1:10)	1 µL*
Milli-Q water	15.75 µL

TABLE 11 | PCR conditions used for ITS amplification.

Locus	**Primers set**	**PCR conditions**				**Reference**
		Denaturation	**Annealing**	**Extension**	**Cycles**	
ITS	ITS5 (forward) NL4 (reverse)	94 ^{o}C, 30 s	50 ^{o}C, 30 s	72 ^{o}C, 90 s	×30	Hilário, 2017

After amplification, the DNA fragments were separated by electrophoresis in 1.5 % agarose gel in 1× TAE (40 mM Tris, pH 7.6; 20 mM Acetic acid; 1 mM EDTA), at 80 V for 60 minutes. It was added 5 µL of each sample and a DNA ladder (GeneRuler DNA Ladder Mix 0.5 µg/µL, 50 µg, Thermo Scientific ™, USA) and applied for each electrophoresis running. After that, the gels were stained with ethidium bromide for 10 minutes, washed in distilled water for 20 minutes and visualised under UV light using a GELDOC XR+ (BioRad, USA), to confirm amplification success.

3.8. PURIFICATION OF THE PCR PRODUCTS

The NZYGelpure kit (Nzytech™, Portugal) was used for amplicons purification and applied according to manufacturer´s instructions. The purified amplification products were sequenced by GATC Biotech (Germany) (see supplementary information S2).

3.9. IDENTIFICATION OF THE FUNGAL ISOLATES

The nucleotide sequences were read and edited using FinchTV v.1.4 (Geospiza Inc. http://www.geospiza.com/finchtv). All sequences were submitted to a first analysis using the Basic Local Alignment Search Tool (BLAST), which compared the sequences against others known sequences in the database. The values resulting from "Query cover" and "Identity" were recorded. Higher query cover and higher identity mean the best match of the sequence and the sequence of database.

This analysis allowed for species/genus assessment of the isolates selected for ITS sequencing, as well as other isolates which presented high similarity with them.

3.10. DIVERSITY ANALYSIS

The richness and the co-occurrence of different genera in the same algae sample (host) were expressed using the Shannon´s *H´* and Simpson diversity indices (Motwani *et al*., 2014; Pielou, 1966). Also, the measure of Pielou´s evenness was assessed to analyse the relative abundance of the different species within a community of the samples (Motwani *et al*., 2014; Pielou, 1966).

All the diversity measures (indicated above) and statistical analyses were performed using R version 3.6.0 (R Core Team, 2019). Default R packages were used for the analyses, unless otherwise mentioned. The richness and diversity (Shannon´s H´ and Simpson´s indices) and Pielou´s evenness indices were calculated using the *vegan* package version 2.5 – 4 (Oksanen *et al*., 2019). In order to find if there are some fungal genera associated with determined factor (sampling site or host) it was used the *multipatt* function from package *indicspecies* version 1.7.6 (De Caceres & Jansen, 2016), using a 10 % of significance level and the parameter "IndVal.g" to correct the unequal group sizes.

The normality and homoscedasticity of variances were assessed for all diversity and evenness indices, using Shapiro-Wilk and Bartlett tests, respectively (p-value > 0.05). Parametric tests (ANOVA) were then used when normality and homoscedasticity of variances were observed. When these conditions were not met, the respective nonparametric tests [Kruskal-Wallis rank sum (R *stats* package; R Core Team, 2019) and post-hoc Kruskal Nemenyi test (*PMCMR* package, version 4.3; Pohlert, 2018)] were performed.

The community-level relationships among samples were assessed using functions from the package *vegan*: the Bray-Curtis dissimilarity index for Community Ecologists (function *vegdist* in R) was used to assess the relationships between samples, through the analysis of a dissimilarity matrix. Then, the PERMANOVA (Permutational Multivariate Analysis of Variance Using Distance Matrices; function *adonis* from *vegan* package in R) was applied to Bray-Curtis results in order to analyse variances between communities according to sampling site or algae host. The data obtained from Bray-Curtis dissimilarity was used to create a Principal Coordinate analysis (PCoA) through the *cmdscale* function (*vegan* package in R). PCoA results in a "graphic bidimensional representation" of distribution of the samples, where are chosen the two axis which more contributed for the data variances (Anderson & Willis, 2003). These two variables with the highest significance were used to perform the axis 1 and 2.

3.11. CHARACTERIZATION OF TWO NEW SPECIES

3.11.1. MOLECULAR ANALYSIS AND PHYLOGENETIC INFERENCE

After the ITS identification, it was noted the presence of five and one isolates belonging to *Cladosporium* and *Hypoxylon* genera, respectively, without an identification to species level. In order to assess the species identification, it was amplified three additional loci.

In addition to ITS, it was performed the partial amplification and sequencing of translation elongation factor 1-α (*tef1-α*) and actin (*actA*) loci for *Cladosporium* isolates (Table 12). For *tef1-α* amplification it was used the primers set EF1-728F/EF1-986R and ACT-512F/ACT-783R were used to amplify the actin gene (Bensch *et al*., 2012; Sandoval-Denis *et al*., 2016). For *tef1-α* amplification, it was performed an initial denaturation at 95 ^{o}C for 3 min, followed by 35 cycles of denaturation temperature of 94 ^{o}C for 30 s, primer annealing at 52 ^{o}C for 30 s and primer extension at 72 ^{o}C

TABLE 12 | Nucleotide sequences of the primers used for actin, *tef1-α* and β-tubulin reactions amplification.

Primers DNA sequences used		
Locus	**Primer**	**DNA sequence (5´→ 3´)**
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC
	ACT-783R	TACGAGTCCTTCTGGCCCAT
tef1-α	EF1-728F	CATCGAGAAGTTCGAGAAGG
	EF1-986R	TACTTGAAGGAACCCTTACC
β-tubulin	T1	AACATGCGTGAGATTGTAAGT
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC

during 45 s, ending with an elongation of 72 ^{o}C for 10 minutes. The PCR conditions for actin gene amplification were the same described by Bensch *et al*. (2012) (Table 13).

For *Hypoxylon* species analysis part of the β-tubulin gene was amplified using the primers set T1/Bt2b (Kuhnert *et al*., 2014) (Table 12). The conditions were as follows: initial denaturation at 95 ^{o}C during 3 min, 35 cycles of primers denaturation at 94 ^{o}C for 30 s, primer annealing at 50 ^{o}C for 30 s and primer extension at 72 ^{o}C during 1 min. After that, it was made one step of elongation at 72 ^{o}C for 10 min (Table 13).

TABLE 13 | PCR conditions used for actin, *tef1-α* and β-tubulin amplifications.

Locus	Primers set	PCR conditions			
		Denaturation	Annealing	Extension	Cycles
Actin	ACT-512F/ACT-783R	94 ^{o}C, 45 s	52 ^{o}C, 30 s	72 ^{o}C, 90 s	×40
tef1-α	EF1-728F/EF1-986R	94 ^{o}C, 30 s	52 ^{o}C, 30 s	72 ^{o}C, 45 s	×35
β-tubulin	T1/Bt2b	94 ^{o}C, 30 s	50 ^{o}C, 30 s	72 ^{o}C, 60 s	×35

Both the PCR solutions were prepared for a final volume of 25 µl: 6.25 µl of NZYTaq 2× Green Master Mix, 1 µL (10 pmol/µL) of each primer used, 15.75 µL of sterile Milli-Q water and 1 µL of DNA template.

The amplification of *tef1-α* and actin genes for *Cladosporium* species and β-tubulin gene for *Hypoxylon* were confirmed by electrophoresis, before their sequencing at GATC Biotech (Cologne, Germany). The sequences obtained were analysed and edited using the FinchTV v.1.4.0 (Geospiza Inc. www.geospiza.com/finchtv), and it was made a BLASTn search against the nucleotide records in the databases (nr/nt). To determine the phylogenetic affiliation of *Cladosporium* species it was added the ITS, *tef1-α* and actin closest matching sequences to the sequence alignment (Table 15); Also, to define the phylogenetic affiliation for *Hypoxylon* species, the closest matching sequences resulted from the BLASTn search using the ITS and tubulin genes were included in the sequence alignment (Table 16).

All the sequences were aligned using the program ClustalX v. 2.1 and the parameters indicated in the Table below (Table 14). The alignments were edited using the BioEdit v.7.2.5, and the phylogenetic analyses were performed in MEGA7 v.7.0. (Kumar *et al*., 2016). All the gaps were included in these analyses. Also, MEGA7 v.7.0 was used to define the most suitable substitution

model for *Cladosporium* and *Hypoxylon* sequences alignment to build the Maximum Likelihood (ML) Tree. To perform the *Cladosporium* tree inference it was used the General Time Reversible model as the heuristic method with 1000 bootstrap replicates. Kimura 2-parameter model was the heuristic model used to build the *Hypoxylon* tree inferences, also using 1000 bootstrap replicates. *Cercospora beticola* and *Creosphaeria sassafras*, were the species chosen as outgroup for *Cladosporium* and *Hypoxylon* alignments, respectively.

TABLE 14 | Parameters defined in ClustalX to perform the sequences alignments.

Alignment parameters	Pairwise Alignment	Multiple Alignment
Gap opening	10	10
Gap extension	0.1	0.2
Transition weight	-	0.5
Delay divergent sequences	-	25 %

3.11.2. GROWTH, MORPHOLOGY AND MICROSCOPIC ANALYSIS

The morphological characteristics were observed using a SMZ1500 stereoscopic microscope and a Nikon Eclipse 80i microscope equipped with differential interference contrast (DIC) (Nikon, Japan). The fungal structures were mounted in a solution of 100 % lactic acid and photographed with a Nikon DSRi1 camera (Nikon, Japan). All fungal structures were measured using the NIS-Elements D program (Nikon, Japan).

CLADOSPORIUM SP. NOV.

The new record belonging to *Cladosporium* genus was incubated for 14 days at 25 ^{o}C, on Petri dishes (55 ø), in triplicate, on oatmeal agar (OA), potato dextrose agar (PDA) and synthetic nutrient-poor agar (SNA) (Sandoval-Denis *et al*., 2016). To characterize the culture colors (obvers and reverse) it was used the color charts of Rayner (Rayner, 1970). The micromorphology of the isolate was carried in SNA incubated at 30 ºC.

Cardinal temperatures of *Cladosporium* sp. nov. were determined by cultivation of the isolate at 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C and 35 ^{o}C, for 14 days.

TABLE 15 | GenBank and culture collection accession numbers for ITS, *tef1-α* and actin of *Cladosporium* species used to infer the phylogeny of this group (*- type species).

Species	Strain	Host/Substrate	Origin	GenBank accession numbers		
				ITS	*tef1-α*	*act*
Cercospora beticola	CBS 116456*	*Beta vulgaris*	Italy	NR_121315	AY840494	AY840458
Cladosporium acalyphae	CBS 125982*	*Acalypha australis*	South Korea	HM147994	HM148235	HM148481
Cladosporium alboflavescens	UTHSC DI-13-225	*Homo sapiens*	USA	LN834420	LN834516	LN834604
Cladosporium angulosum	CBS 140692*	*Homo sapiens*	USA	LN834425	LN834521	LN834609
Cladosporium angustisporum	CBS 125983*	*Alloxylon wickhamii*	Australia	HM147995	HM148236	HM148482
Cladosporium angustiterminale	CBS 140480*	*Banksia grandis*	Australia	KT600379	KT600476	KT600575
Cladosporium anthropophilum	CBS 140685*	*Homo sapiens*	USA	LN834437	LN834533	LN834621
	CBS 117483	-	USA	HM148007	HM148248	HM148494
Cladosporium asperulatum	CBS 126340*	*Protea susannae*	Portugal	HM147998	HM148239	HM148485
Cladosporium australiense	CBS 125984*	*Eucalyptus moluccana*	Australia	HM147999	HM148240	HM148486
Cladosporium austroafricanum	CBS 140481*	Leaf litter	South Africa	KT600381	KT600478	KT600577
Cladosporium chalastosporoides	CBS 125985*	*Teratosphaeria proteae-arboreae*	South Africa	HM148001	HM148242	HM148488
Cladosporium chubutense	CBS 124457*	*Pinus ponderosa*	Argentina	FJ936158	FJ936161	FJ936165
Cladosporium cladosporioides	CBS 112388*	"Indoor air"	Germany	HM148003	HM148244	HM148490
	CBS 113738	Grape	USA	HM148004	HM148245	HM148491
	CPC 14292	Soil from pea field	Denmark	HM148046	HM148287	HM148533
	CBS 101367	-	Brazil	HM148002	HM148243	HM148489
	CBS 113739	Crested wheat grass	USA	HM148005	HM148246	HM148492
	CBS 113740	Berry of grape	USA	HM148006	HM148247	HM148493
	CBS 126341	*Spinacia oleracea*	USA	HM148009	HM148250	HM148496
	CBS 143.35	*Pisum sativum*	South Africa	HM148011	HM148252	HM148498

	CBS 144.35	-	USA	HM148012	HM148253	HM148499
	CBS 145.35	*Pisum sativum*	Germany	HM148013	HM148254	HM148500
	CPC 11120	*Viola mandshurica*	South Korea	HM148017	HM148258	HM148504
	CPC 11121	*Celosia cristata*	South Korea	HM148018	HM148259	HM148505
	CPC 11161	*Eucalyptus* sp.	India	HM148022	HM148263	HM148509
	CMG 43	***Fucus spiralis***	**Portugal**	**MN053016**	**MN066642**	**MN066637**
Cladosporium colocasiae	CBS 386.64*	*Colocasia esculenta*	Taiwan	NR_119840	HM148310	HM148555
	CBS 119542	*Colocasia esculenta*	Japan	HM148066	HM148309	HM148554
Cladosporium colombiae	CBS 274.80B*	*Cortaderia* sp.	Colombia	FJ936159	FJ936163	FJ936166
Cladosporium crousii	UTHSC DI-13-247*	*Homo sapiens*	USA	LN834431	LN834527	LN834615
Cladosporium cucumerinum	CBS 171.52*	*Cucumis sativus*	Netherlands	HM148072	HM148316	HM148561
Cladosporium delicatulum	CBS 126344	*Tilia cordata*	Germany	HM148081	HM148325	HM148570
	CBS 126342	"Indoor air"	Denmark	HM148079	HM148323	HM148568
Cladosporium exasperatum	CBS 125986*	*Eucalyptus tintinnans*	Australia	HM148090	HM148334	HM148579
Cladosporium exile	CBS 125987*	*Phyllactinia guttata*	USA	HM148091	HM148335	HM148580
Cladosporium flabelliforme	CBS 126345*	*Melaleuca cajuputi*	Australia	HM148092	HM148336	HM148581
Cladosporium flavovirens	CBS 140462*	*Homo sapiens*	USA	LN834440	LN834536	LN834624
Cladosporium funiculosum	CBS 122129*	*Phaseolus chrysanthos*	Japan	NR_119845	HM148338	HM148583
Cladosporium gamsianum	CBS 125989*	*Strelitzia* sp.	South Africa	HM148095	HM148339	HM148584
Cladosporium globisporum	CBS 812.96*	"Meat stamp"	Sweden	HM148096	HM148340	HM148585
Cladosporium grevilleae	CBS 114271*	*Grevillea* sp. (leaves)	Australia	JF770450	JF770472	JF770473
Cladosporium hillianum	CBS 125988*	Leaf mold of *Typha orientalis*	New Zealand	HM148097	HM148341	HM148586
Cladosporium inversicolor	CBS 401.80*	*Triticum aestivum* (leaf)	Netherlands	HM148101	HM148345	HM148590
Cladosporium ipereniae	CBS 140483*	*Puya* sp.	Chile	KT600394	KT600491	KT600589
Cladosporium iranicum	CBS 126346*	*Citrus sinensis* (leaf)	Iran	HM148110	HM148354	M148599
Cladosporium licheniphilum	CBS 125990*	*Phaeophyscia orbicularis* and Physcia sp.	Germany	HM148111	HM148355	HM148600
Cladosporium longicatenatum	CBS 140485*	"Unknown" plant	Australia	KT600403	KT600500	KT600598

Cladosporium lycoperdinum	CBS 126347	*Apiosporina morbosa*	Canada	HM148112	HM148356	HM148601
	CBS 574.78C	*Aureobasidium caulivorum*	Russia	HM148115	HM148359	HM148604
Cladosporium montecillanum	CBS 140486*	Pine *needles*	Mexico	KT600406	KT600504	KT600602
Cladosporium myrtacearum	CBS 126350*	*Corymbia foelscheana*	Australia	HM148117	HM148361	HM148606
Cladosporium needhamense	CBS 143359*	"Indoor air"	USA	MF473142	MF473570	MF473991
Cladosporium neerlandicum	CBS 143360*	-	Netherlands	KP701887	KP701764	KP702010
Cladosporium neopsychrotolerans	CGMCC 3.18031	Rhizosphere soil of *Saussurea involucrate*	China	KX938383	KX938400	KX938366
Cladosporium oxysporum	CBS 125991*	Soil (terracotta gravene)	China	HM148118	HM148362	HM148607
	CBS 126351	"Indoor air"	Venezuela	HM148119	HM148363	HM148608
Cladosporium paracladosporioides	CBS 171.54*	-	-	HM148120	HM148364	HM148609
Cladosporium parapenidielloides	CBS 140487*	*Eucalyptus* sp.	Australia	KT600410	KT600508	KT600606
Cladosporium perangustum	CBS 125996*	*Cussonia* sp.	South Africa	HM148121	HM148365	HM148610
	CBS 126365	*Phyllactinia guttata*	USA	HM148123	HM148367	HM148612
	CPC 13870	*Teratosphaeria fibrillosa*	South Africa	HM148142	HM148386	HM148631
	CPC 12216	*Morus rubra*	Germany	HM148135	HM148379	HM148624
	CPC 13730	*Protea caffra*	South Africa	HM148140	HM148384	HM148629
	CMG 44	***Enteromorpha* sp.**	**Potugal**	**MN053017**	**MN066643**	**MN066638**
	CMG 45	**Filamentous Green alga**	**Potugal**	**MN053019**	**MN066645**	**MN066640**
	CMG 46	***Fucus* sp.**	**Potugal**	**MN053020**	**MN066646**	**MN066641**
Cladosporium phaenocomae	CBS 128769*	*Phaenocoma prolifera*	South Africa	JF499837	JF499875	JF499881
Cladosporium phyllactiniicola	CBS 126355*	*Phyllactinia guttata*	USA	HM148153	HM148397	HM148642
Cladosporium phyllophilum	CBS 125992*	*Taphrina* sp.	Germany	HM148154	HM148398	HM148643
Cladosporium pini-ponderosae	CBS 124456*	*Pinus ponderosa*	Argentina	FJ936160	FJ936164	FJ936167
Cladosporium pseudochalastosporoides	CBS 140490*	Pine needles	Mexico	KT600415	KT600513	KT600611

Cladosporium pseudocladosporioides	CBS 125993*	"Outside air"	Netherlands	HM148158	HM148402	HM148647
	CBS 667.80	*Malus sylvestris*	Italy	HM148165	HM148409	HM148654
Cladosporium rectoides	CBS 125994*	*Vitis flexuosa*	South Korea	HM148193	HM148438	HM148683
Cladosporium rubrum	**MUM 19.39/CMG 28***	***Enteromorpha* sp.**	**Portugal**	**MN053018**	**MN066644**	**MN066639**
Cladosporium rugulovarians	CBS 140495	Leaf sheaths of unidentified Poaceae	Brazil	KT600459	KT600558	KT600656
Cladosporium scabrellum	CBS 126358*	*Ruscus hypoglossum*	Slovenia	HM148195	HM148440	HM148685
Cladosporium silenes	CBS 109082	*Silene uniflora*	UK	EF679354	EF679429	EF679506
Cladosporium subuliforme	CBS 126500*	*Chamaedorea metallica*	Thailand	HM148196	HM148441	HM148686
Cladosporium tenuissimum	CPC 14253*	*Lagerstroemia* sp.	USA	NR_119855	HM148442	HM148687
Cladosporium varians	CBS 126362*	*Catalpa bungei*	Russia	HM148224	HM148470	HM148715
Cladosporium verrucocladosporioides	CBS 126363*	*Rhus chinensis*	South Korea	HM148226	HM148472	HM148717
Cladosporium xantochromaticum	CBS 140691*	*Homo sapiens* (bronchoalveolar lavage fluid)	USA	LN834415	LN834511	LN834599
	CBS 126364	*Erythrophleum chlorostachys*	Australia	HM148122	HM148366	HM148611
Cladosporium xylophilum	CBS 125997*	*Picea abies*	Russia	HM148230	HM148476	HM148721

HYPOXYLON SP. NOV.

The isolate belonging to *Hypoxylon* genus was cultivated on PDA and OA (triplicate in both the media) on Petri dishes (55 ø), for 14 days at 25 ^{o}C temperature (in the dark), in order to analyse the cultural and micromorphological characterization (Ulloa-Benítez *et al*., 2016). Colony colours (obvers and reverse) were determined based on the color charts of Rayner (Rayner, 1970).

Cardinal temperatures of *Hypoxylon* sp. nov. were determined by cultivation of the isolate at 5 ^{o}C, 10 ^{o}C, 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C and 35 ^{o}C, for 14 days.

TABLE 16 | GenBank and culture collection accession numbers for ITS and β-tubulin of *Hypoxylon* species used to infer the phylogeny of this group (*- type species).

Species	Strain	Host/Substrate	Origin	GenBank accession numbers	
				ITS	*tub2*
Hypoxylon aveirense	**MUM 19.40/CMG 29***	***Fucus* sp.**	**Portugal**	**MN053021**	**MN066636**
Hypoxylon begae	BCRC 34051	*Myoporum sandwicense* (branch)	Hawaii	JN660820	AY951704
Hypoxylon griseobrunneum	CBS 331.73*	Soil from *Piper betle* orchard	India	KC968918	KC977303
	BCRC 34050	Wood	Mexico	JN660819	AY951703
	CBS 129346	-	Guadeloupe	KC968928	KC977281
	MUCL 53754	-	Martinique	KC968909	KC977289
	CBS 117742	Halfburned piece of wood, in brush pile	Ecuador	KC968917	KC977271
Hypoxylon vinosopulvinatum	BCRC 34101*	Wood	Taiwan	JQ009321	AY951761
Hypoxylon addis	MUCL 52797*		Ethiopia	KC968931	KC977287
Hypoxylon cercidicola	CBS 119009	Fraxinus excelsior	France	KU683766	KU684189
Hypoxylon hypomiltum	MUCL 51845	-	Guadeloupe	KY610403	KX271249
Hypoxylon isabellinum	CBS 129035*	-	Martinique	KC968935	KC977295
Hypoxylon munkii	MUCL 53315	-	Martinique	KC968912	KC977294
Hypoxylon musceum	MUCL 53765	-	Guadeloupe	KC968926	KC977280
Hypoxylon ochraceum	MUCL 54625*	-	Martinique	KC968937	KC977300
Hypoxylon perforatum	CBS 115281	Fraxinus excelsior	France	KY610391	KX271250
Hypoxylon petriniae	CBS 114746*	*Fraxinus excelsior*	France	KY610405	KX271274
Hypoxylon rubiginosum	MUCL 52887*	-	Germany	KC477232	KY624311
Hypoxylon samuelsii	MUCL 51843*	-	Guadeloupe	KC968916	KC977286
Hypoxylon investiens	CBS 118185	-	Ecuador	KC968924	KC977269
	MUCL 53307	-	Martinique	KC477239	KC977293
	CBS 118183	-	Malaysia	KC968925	KC977270
Hypoxylon lateripigmentum	CBS 129031*	-	Martinique	KC968933	KC977290
Hypoxylon pulicicidum	CBS 122622*	-	Martinique	JX183075	JX183072
Creosphaeria sassafras	CBS 119001	*Lauraceae* sp.	Spain	KU683754	KU684126

4. RESULTS & DISCUSSION

4.1. SAMPLING AND FUNGAL ISOLATION

From the 7 sites chosen in Ria de Aveiro, 26 samples were collected from seaweeds belonging to genera *Ulva*, *Fucus*, *Enteromorpha*, *Gracilaria*, *Cladophora*, *Ceramium*, *Codium* and *Scinaia*. Four algae samples could not be identified at genus level (samples 2,4, 13 and 21). It is important to

TABLE 17| Number of fungal isolates obtained from each algae sample. Red alga 1 – algae samples belonging to the same genus. *- unknown algae genus.

	Algae samples		Fungi
Local	**Algae identification**		**Number of isolates**
A	1	*Ulva* sp.	12
	2	Red algae 1*	12
	3	*Ulva* sp.	12
	4	Red algae 1*	18
	5	*Fucus* sp.	15
	6	*Fucus* sp.	11
B	7	*Fucus spiralis*	21
	8	*Fucus spiralis*	18
	9	*Enteromorpha* sp.	20
	10	*Fucus* sp.	14
	11	*Fucus* sp.	29
	12	*Fucus* sp.	24
C	13	Filamentous Green algae*	22
	14	*Fucus* sp.	24
	15	*Gracilaria gracilis*	10
	16	*Ulva* sp.	13
	17	*Ulva* sp.	34
D	18	*Cladophora* sp.	35
	19	*Cladophora* sp.	20
E	20	*Ceramium* sp.	10
	21	Red algae*	21
	22	*Ulva* sp.	40
	23	*Enteromorpha intestinalis*	26
F	24	*Fucus* sp.	9
G	25	*Codium* sp.	8
	26	*Scinaia* sp.	8
Total		26 samples	486 isolates

highlight that the algae samples collected depended of the species available in each site. The algae collection and respective fungal community isolation resulted in a total of 486 isolates (Table 17).

Considering Table 17 it is possible to conclude that the green algae are the hosts with the highest number of fungal isolates obtained, as happen with the hosts belonging to *Ulva* and *Cladophora* genera. On the other hand, in the red algae it was obtained the lowest fungal isolates, namely, in the case of *Scinaia* alga.

4.2. PCR FINGERPRINTING AND ITS IDENTIFICATION

All 486 isolates were characterized by MSP-PCR using the GTG_5 sequence (see supplementary Figure S3). After electrophoresis and visualization under UV light, the DNA profiles obtained were analysed based on their similarity and resulted in a dendrogram. After analysis, 213 representative isolates were chosen for ITS amplification and further sequencing. Using the Standard Nucleotide BLAST, it was possible to obtain a primary identification of fungal isolates (Table 18).

TABLE 18 | Results from ITS Blastn analysis against Genbank database. The identity was registered in percentage. Gen. nov. – new genus record belonging to Didymosphaeriaceae family.

Local	Seaweed	Isolates identification		Phylogenetic affiliation	Identity (%)
A	1	A_1_F1	1	*Penicillium* sp.	99 %
		A_1_F2	2	*Leptobacillium leptobactrum*	100 %
		A_1_F3	3	*Paraphaeosphaeria* sp.	99 %
		A_1_F4	4	*Leptobacillium leptobactrum*	100 %
		A_1_F5	5	*Leptobacillium leptobactrum*	100 %
		A_1_F6	6	*Leptobacillium leptobactrum*	100 %
		A_1_F7	7	*Leptobacillium leptobactrum*	100 %
		A_1_F9	**8**	*Simplicillium lamellicola*	100 %
		A_1_F10	9	*Leptobacillium leptobactrum*	100 %
		A_1_F11	10	*Leptobacillium leptobactrum*	100 %
		A_1_F14	**11**	*Leptobacillium leptobactrum*	100 %
		A_1_F15	12	*Leptobacillium leptobactrum*	100 %
	2	A_2_F1	**13**	*Fusarium* sp.	100 %
		A_2_F2	14	*Botrytis* sp.	100 %
		A_2_F4	**16**	*Penicillium* sp.	100 %
		A_2_F5	17	*Cladosporium cladosporioides*	100 %
		A_2_F7	18	*Paraphaeosphaeria* sp.	99 %
		A_2_F8	19	*Leptobacillium leptobactrum*	100 %
		A_2_F11	20	*Paraphaeosphaeria sp*	99 %
		A_2_F13	**21**	*Aspergillus niger*	100 %
		A_2_F15	22	*Paraphaeosphaeria* sp.	99 %
		A_2_F16	**23**	*Cladosporium* sp.	99 %
		A_2_F17	24	*Leptobacillium leptobactrum*	100 %
		A_2_F18	25	*Cladosporium* sp.	99 %

3	A_3_F1	**28**	*Cladopsporium* sp.	100 %
	A_3_F2	29	*Leptobacillium leptobactrum*	100 %
	A_3_F3	30	*Leptobacillium leptobactrum*	100 %
	A_3_F4	**31**	*Leptobacillium leptobactrum*	100 %
	A_3_F6	32	*Leptobacillium leptobactrum*	100 %
	A_3_F7	33	*Leptobacillium leptobactrum*	100 %
	A_3_F8	34	*Leptobacillium leptobactrum*	100 %
	A_3_F9	35	*Leptobacillium leptobactrum*	100 %
	A_3_F10	36	*Leptobacillium leptobactrum*	100 %
	A_3_F12	37	*Leptobacillium leptobactrum*	100 %
	A_3_F16	38	*Leptobacillium leptobactrum*	100 %
	A_3_F17	39	*Leptobacillium leptobactrum*	100 %
4	A_4_F1	**40**	*Fusarium* sp.	99 %
	A_4_F2	**41**	*Penicillium expansum*	99 %
	A_4_F4	**42**	*Penicillium grabrum*	99 %
	A_4_F5	43	Gen. nov.	-
	A_4_F6	44	*Parasarocladium radiatum*	97 %
	A_4_F7	45	*Diaporthe* sp.	99 %
	A_4_F8	**46**	*Stagonospora* sp.	99 %
	A_4_F9	47	*Leptobacillium leptobactrum*	100 %
	A_4_F10	48	*Leptobacillium leptobactrum*	100 %
	A_4_F11	**49**	*Cladosporium* sp.	99 %
	A_4_F12	50	*Leptobacillium leptobactrum*	100 %
	A_4_F14	51	*Leptobacillium leptobactrum*	100 %
	A_4_F15	52	*Leptobacillium leptobactrum*	100 %
	A_4_F16	**53**	*Paraphaeosphaeria* sp.	99 %
	A_4_F17	54	*Fusarium* sp.	99 %
	A_4_F18	55	*Leptobacillium leptobactrum*	100 %
	A_4_F19	56	*Fusarium* sp.	99 %
	A_4_F20	**57**	*Diaporthe* sp.	99 %
5	A_5_F1	58	*Botrytis eucalypti*	100 %
	A_5_F5	61	*Cladosporium* sp.	100 %
	A_5_F6	62	*Leptobacillium leptobactrum*	100 %
	A_5_F7	**63**	*Cladosporium* sp.	100 %
	A_5_F9	**64**	*Cladosporium* sp.	99 %
	A_5_F10	65	*Acremonium potronii*	99 %
	A_5_F11	66	*Penicillium brevicompactum*	99 %
	A_5_F12	67	*Leptobacillium leptobactrum*	100 %
	A_5_F13	68	*Cladosporium* sp.	100 %
	A_5_F14	69	*Parasarocladium radiatum*	96 %
	A_5_F15	70	*Botrytis eucalypti*	100 %
	A_5_F16	**71**	*Parasarocladium* sp.	96 %
	A_5_F17	72	*Botrytis eucalypti*	100 %
	A_5_F18	73	*Paraphaeosphaeria* sp.	99 %
	A_5_F19	74	*Leptobacillium leptobactrum*	100 %
6	A_6_F1	**75**	*Penicillium brocae*	100 %
	A_6_F2	76	*Parasarocladium radiatum*	97 %

		A_6_F3	77	*Paradendryphiella salina*	100 %
		A_6_F4	78	*Cladosporium cladosporioides*	100 %
		A_6_F6	**79**	*Aspergillus fumigatus*	100 %
		A_6_F7	80	*Paraphaeosphaeria* sp.	99 %
		A_6_F9	81	*Leptobacillium leptobactrum*	100 %
		A_6_F11	82	*Leptobacillium leptobactrum*	100 %
		A_6_F12	**83**	*Paradendryphiella salina*	100 %
		A_6_F13	84	*Paraphaeosphaeria* sp.	99 %
		A_6_F15	85	*Paraphaeosphaeria* sp.	99 %
B	7	B_7_F1	**86**	*Trichoderma alni*	99 %
		B_7_F2	87	*Cladosporium* sp.	100 %
		B_7_F3	**88**	*Epicoccum nigrum*	100 %
		B_7_F4	**89**	*Cladosporium cladosporioides*	99 %
		B_7_F6	90	*Fusarium solani*	100 %
		B_7_F7	**91**	*Acremonium potronii*	99 %
		B_7_F8	**92**	*Epicoccum nigrum*	99 %
		B_7_F9	**93**	*Aspergillus* sp.	99 %
		B_7_F10	94	*Alternaria* sp.	100 %
		B_7_F11	95	*Fusarium solani*	100 %
		B_7_F12	96	*Fusarium solani*	100 %
		B_7_F13	**97**	*Aspergillus* sp.	100 %
		B_7_F14	98	*Cladosporium* sp.	100 %
		B_7_F15	99	*Epicoccum nigrum*	100 %
		B_7_F16	**100**	*Diaporthe pyracanthae*	99 %
		B_7_F17	**101**	*Leptobacillium leptobactrum*	99 %
		B_7_F18	**102**	*Leptobacillium leptobactrum*	100 %
		B_7_F19	**103**	*Cladosporium* sp.	98 %
		B_7_F20	**104**	*Epicoccum nigrum*	100 %
		B_7_F21	**105**	*Cladosporium* sp.	99 %
		B_7_F22	106	*Exophiala oligosperma*	100 %
	8	B_8_F1	**107**	*Cladosporium* sp.	100 %
		B_8_F2	**108**	*Arthrinium kogelbergense*	100 %
		B_8_F3	**109**	*Mortierella* sp.	99 %
		B_8_F4	110	*Cladosporium* sp.	99 %
		B_8_F5	111	*Cladosporium* sp.	99 %
		B_8_F6	112	*Cladosporium* sp.	99 %
		B_8_F7	113	*Mortierella* sp.	99 %
		B_8_F8	**114**	*Botrytis cinerea*	99 %
		B_8_F9	115	*Cladosporium* sp.	99 %
		B_8_F10	**116**	*Cladosporium* sp.	99 %
		B_8_F11	**117**	*Penicillium brevicompactum*	99 %
		B_8_F12	**118**	*Cladosporium* sp.	99 %
		B_8_F13	119	*Cladosporium* sp.	99 %
		B_8_F14	120	*Cladosporium cladosporioides*	100 %
		B_8_F16	**121**	*Penicillium brevicompactum*	99 %
		B_8_F17	122	*Cladosporium* sp.	100 %
		B_8_F18	123	*Cladosporium* sp.	100 %

	B_8_F19	124	*Leptobacillium leptobactrum*	100 %
9	B_9_F1	**125**	*Cladosporium* sp.	99 %
	B_9_F2	**126**	*Cladosporium* sp.	100 %
	B_9_F3	127	*Cladosporium* sp.	99 %
	B_9_F4	**128**	Gen. nov.	-
	B_9_F6	**129**	*Pithomyces chartarum*	100 %
	B_9_F8	**130**	Gen. nov.	-
	B_9_F9	131	*Epicoccum nigrum*	99 %
	B_9_F10	**132**	*Cladosporium* sp.	100 %
	B_9_F11	**133**	*Cladosporium* sp.	100 %
	B_9_F12	134	*Epococcum nigrum*	99 %
	B_9_F13	**135**	*Cladosporium* sp.	100 %
	B_9_F14	**136**	*Alternaria alternata*	100 %
	B_9_F15	**137**	*Cladosporium* sp.	100 %
	B_9_F16	**138**	*Phaeosphaeria* sp.	99 %
	B_9_F18	**139**	*Emericellopsis* sp.	97 %
	B_9_F19	140	*Cladosporium cladosporioides*	100 %
	B_9_F20	**141a**	*Cladosporium* sp.	99 %
		141b	*Cladosporium* sp.	99 %
	B_9_F22	**142**	*Cladosporium* sp.	99 %
	B_9_F23	143	*Cladosporium* sp.	99 %
10	B_10_F1	144	*Cladosporium* sp.	100 %
	B_10_F2	**145**	*Cladosporium* sp.	99 %
	B_10_F3	146	*Cladosporium* sp.	100 %
	B_10_F4	**147**	*Stemphylium vesicarium*	100 %
	B_10_F5	**148**	*Alternaria* sp.	99 %
	B_10_F6	**149**	*Botryosporium longibrachiatum*	100 %
	B_10_F7	150	*Alternaria* sp.	100 %
	B_10_F8	**151**	*Alternaria* sp.	100 %
	B_10_F9	**152**	*Neocamarosporium* sp.	98 %
	B_10_F10	153	*Botryosporium longibrachiatum*	99 %
	B_10_F12	154	*Stemphylium globuliferum*	100 %
	B_10_F13	155	*Botryosporium longibrachiatum*	99 %
	B_10_F14	156	*Botryosporium longibrachiatum*	100 %
	B_10_F15	157	*Botryosporium longibrachiatum*	99 %
11	B_11_F1	**158**	*Alternaria* sp.	99 %
	B_11_F3	160	*Fusarium solani*	100 %
	B_11_F4	**161**	*Botrytis eucalypti*	100 %
	B_11_F6	**162**	*Cladosporium* sp.	100 %
	B_11_F7	163	*Cladosporium* sp.	100 %
	B_11_F8	164	*Alternaria* sp.	100 %
	B_11_F9	165	*Pestalotiopsis* sp.	99 %
	B_11_F10	166	*Pestalotiopsis* sp.	99 %
	B_11_F11	167	*Cladosporium* sp.	100 %
	B_11_F12	**168**	*Alternaria* sp.	100 %
	B_11_F14	**170**	*Epicoccum nigrum*	100 %
	B_11_F15	171	*Penicillium brevicompactum*	99 %

		B_11_F16	172	*Fusarium solani*	100 %
		B_11_F17	**173**	*Epicoccum* sp.	99 %
		B_11_F18	174	*Alternaria* sp.	99 %
		B_11_F19	**175**	*Alternaria* sp.	99 %
		B_11_F20	**176**	*Alternaria* sp.	99 %
		B_11_F21	177	*Diaporthe* sp.	99 %
		B_11_F22	178	*Fusarium solani*	100 %
		B_11_F24	179	*Epococcum nigrum*	99 %
		B_11_F25	**180**	*Epococcum nigrum*	99 %
		B_11_F26	**181**	*Penicillium* sp.	100 %
		B_11_F27	**182**	*Alternaria* sp.	99 %
		B_11_F28	**183**	*Botryosporium longibrachiatum*	99 %
		B_11_F29	**184**	*Hypoxylon* sp.	99 %
		B_11_F30	185	*Cladosporium* sp.	100 %
		B_11_F31	186	*Fusarium solani*	100 %
		B_11_F32	**187**	*Alternaria infectoria*	99 %
		B_11_F33	**188**	*Cladosporium* sp.	99 %
	12	B_12_F1	189	*Cladosporium cladosporioides*	99 %
		B_12_F2	**190**	*Cladosporium* sp.	100 %
		B_12_F3	191	Gen. nov.	-
		B_12_F4	**192**	*Penicillium* sp.	100 %
		B_12_F5	**193**	*Penicillium* sp.	99 %
		B_12_F6	**194**	*Cladosporium cladosporioides*	99 %
		B_12_F7	**195**	*Periconia* sp.	100 %
		B_12_F8	196	*Cladosporium* sp.	100 %
		B_12_F9	197	*Cladosporium* sp.	100 %
		B_12_F10	**198**	*Cladosporium* sp.	99 %
		B_12_F11	**199**	*Cladosporium* sp.	100 %
		B_12_F12	200	*Cladosporium cladosporioides*	100 %
		B_12_F13	**201**	*Cladosporium* sp.	100 %
		B_12_F14	202	*Cladosporium* sp.	99 %
		B_12_F15	203	*Cladosporium* sp.	99 %
		B_12_F16	**204**	*Talaromyces cecidicola*	99 %
		B_12_F17	205	*Cladosporium* sp.	99 %
		B_12_F18	206	*Stagonospora* sp.	99 %
		B_12_F19	207	*Cladosporium* sp.	100 %
		B_12_F20	**208**	*Lecanicillium* sp.	99 %
		B_12_F21	**209**	*Penicillium* sp.	100 %
		B_12_F22	**210**	*Periconia* sp.	100 %
		B_12_F23	**211**	*Phaeosphaeria halima*	100 %
		B_12_F24	212	*Cladosporium* sp.	99 %
C	13	C_13_F1	**213**	*Cladosporium cladosporioides*	100 %
		C_13_F2	**214**	*Cladosporium* sp.	100 %
		C_13_F3	215	*Cladosporium* sp.	100 %
		C_13_F4	**216**	*Fusarium solani*	100 %
		C_13_F5	217	*Cladosporium* sp.	99 %

	C_13_F6	218	*Botrytis* sp.	100 %
	C_13_F7	219	*Cladosporium cladosporioides*	100 %
	C_13_F8	220	*Emericellopsis pallida*	100 %
	C_13_F9	221	*Cladosporium* sp.	99 %
	C_13_F10	**222**	*Cladosporium cladosporioides*	100 %
	C_13_F11	**223**	*Cladosporium* sp.	99 %
	C_13_F12	**224**	*Acremonium potronii*	99 %
	C_13_F13	**225**	*Stemphylium globuliferum*	100 %
	C_13_F14	226	*Cladosporium* sp.	99 %
	C_13_F15	227	*Cladosporium cladosporioides*	100 %
	C_13_F16	**228**	*Acremonium potronii*	99 %
	C_13_F17	**229**	*Epicoccum nigrum*	100 %
	C_13_F18	230	*Acremonium potronii*	99 %
	C_13_F20	231	*Cladosporium cladosporioides*	100 %
	C_13_F21	232	*Cladosporium* sp.	99 %
	C_13_F23	**234**	*Cladosporium* sp.	100 %
	C_13_F24	**235**	*Botrytis* sp.	100 %
14	C_14_F1	**236**	*Cladosporium* sp.	100 %
	C_14_F2	**237**	*Acremonium potronii*	99 %
	C_14_F3	**238**	*Stagonospora* sp.	99 %
	C_14_F4	239	*Cladosporium* sp.	100 %
	C_14_F5	240	*Cladosporium* sp.	99 %
	C_14_F6	241	*Cladosporium* sp.	99 %
	C_14_F7	**242**	*Acremonium potronii*	99 %
	C_14_F8	243	*Fusarium solani*	100 %
	C_14_F9	**244**	*Cladosporium* sp.	100 %
	C_14_F10	**245**	*Parasarocladium radiatum*	97 %
	C_14_F11	246	*Stagonospora* sp.	99 %
	C_14_F12	247	*Cladosporium cladosporioides*	100 %
	C_14_F13	**248**	*Stemphylium vesicarium*	99 %
	C_14_F14	249	*Stagonospora* sp.	99 %
	C_14_F15	250	*Cladosporium* sp.	100 %
	C_14_F16	251	*Stagonospora* sp.	99 %
	C_14_F17	252	*Stagonospora* sp.	99 %
	C_14_F18	253	*Penicillium brevicompactum*	99 %
	C_14_F19	**254**	*Phaeosphaeria spartinicola*	99 %
	C_14_F21	255	*Exophiala oligosperma*	100 %
	C_14_F23	256	*Stagonospora* sp.	99 %
	C_14_F24	**257**	*Stagonospora* sp.	99 %
	C_14_F25	**258**	*Phaeosphaeria halima*	99 %
	C_14_F26	**259**	*Stemphylium vesicarium*	99 %
15	C_15_F1	260	*Acremonium potronii*	99 %
	C_15_F2	**261**	*Trichoderma koningii*	100 %
	C_15_F4	**262**	*Acremonium potronii*	99 %
	C_15_F5	**263**	*Cladosporium cladosporioides*	100 %
	C_15_F6	**264**	Gen. nov.	-
	C_15_F7	**265**	*Emericellopsis maritima*	99 %

	C_15_F8	**266**	*Penicillium brevicompactum*	99 %
	C_15_F9	267	*Acremonium potronii*	99 %
	C_15_F10	268	*Penicillium brevicompactum*	99 %
	C_15_F11	269	*Penicillium brevicompactum*	99 %
16	C_16_F1	270	*Emericellopsis maritima*	99 %
	C_16_F2	**271**	*Acremonium potronii*	99 %
	C_16_F3	**272**	Gen. nov.	-
	C_16_F4	**273**	*Cladosporium* sp.	100 %
	C_16_F5	274	Gen. nov.	-
	C_16_F6	**275**	*Emericellopsis pallida*	99 %
	C_16_F7	276	*Acremonium potronii*	99 %
	C_16_F8	**277**	*Acremonium potronii*	99 %
	C_16_F9	278	*Acremonium potronii*	99 %
	C_16_F10	279	*Trichoderma* sp.	100 %
	C_16_F11	**280**	*Parasarocladium radiatum*	96 %
	C_16_F12	**281**	*Acremonium fuci*	99 %
	C_16_F13	**282**	*Cladosporium cladosporioides*	98 %
17	C_17_F1	**283**	*Cladosporium* sp.	99 %
	C_17_F2	**284**	*Penicillium brevicompactum*	99 %
	C_17_F3	**285**	*Stagonospora* sp.	99 %
	C_17_F4	286	*Penicillium brevicompactum*	99 %
	C_17_F5	287	*Penicillium brevicompactum*	99 %
	C_17_F6	288	*Acremonium potronii*	99 %
	C_17_F7	**289**	*Emericellopsis* sp.	98 %
	C_17_F8	**290**	Gen. nov.	-
	C_17_F9	**291**	*Alternaria* sp.	99 %
	C_17_F10	**292**	*Alternaria burnsii*	100 %
	C_17_F11	**293**	*Fusarium oxysporum*	99 %
	C_17_F12	294	*Fusarium solani*	100 %
	C_17_F13	295	*Fusarium solani*	100 %
	C_17_F14	**296**	*Cladosporium* sp.	100 %
	C_17_F15	**297**	*Alternaria* sp.	100 %
	C_17_F17	**298**	*Alternaria* sp.	99 %
	C_17_F18	299	Gen. nov.	-
	C_17_F19	**300**	*Fusarium solani*	100 %
	C_17_F20	301	*Cladosporium* sp.	99 %
	C_17_F21	**302**	*Cladosporium* sp.	100 %
	C_17_F22	**303**	*Cladosporium* sp.	100 %
	C_17_F23	304	*Cladosporium* sp.	99 %
	C_17_F24	305	*Cladosporium* sp.	99 %
	C_17_F25	**306**	*Cladosporium cladosporioides*	99 %
	C_17_F26	307	*Cladosporium* sp.	100 %
	C_17_F27	308	*Epicoccum nigrum*	100 %
	C_17_F28	**309**	*Pestalotiopsis* sp.	99 %
	C_17_F29	**310**	*Epicoccum nigrum*	100 %
	C_17_F30	311	*Cladosporium* sp.	100 %
	C_17_F32	313	*Alternaria* sp.	100 %

		C_17_F33	314	*Alternaria* sp.	100 %
		C_17_F34	315	*Alternaria* sp.	100 %
		C_17_F35	**316**	*Cladosporium* sp.	100 %
		C_17_F36	**317**	*Alternaria* sp.	100 %
D	18	D_18_F1	**318**	*Cladosporium* sp.	100 %
		D_18_F2	319	*Cladosporium* sp.	100 %
		D_18_F3	**320**	*Alternaria* sp.	99 %
		D_18_F4	**321**	*Cladosporium* sp.	100 %
		D_18_F5	322	*Stagonospora* sp.	99 %
		D_18_F6	323	*Alternaria alternata*	100 %
		D_18_F7	**324**	*Cladosporium* sp.	100 %
		D_18_F8	325	*Alternaria* sp.	100 %
		D_18_F9	326	*Alternaria* sp.	100 %
		D_18_F10	327	*Cladosporium* sp.	100 %
		D_18_F11	328	*Parasarocladium radiatum*	97 %
		D_18_F12	**329**	*Aspergillus* sp.	100 %
		D_18_F13	330	*Stagonospora* sp.	99 %
		D_18_F14	331	*Stagonospora* sp.	99 %
		D_18_F15	332	*Cladosporium* sp.	100 %
		D_18_F16	333	*Stagonospora* sp.	99 %
		D_18_F17	334	*Cladosporium* sp.	100 %
		D_18_F18	335	*Alternaria* sp.	100 %
		D_18_F19	336	*Alternaria* sp.	100 %
		D_18_F20	**337**	*Cladosporium* sp.	100 %
		D_18_F21	338	*Cladosporium* sp.	100 %
		D_18_F22	339	*Cladosporium* sp.	100 %
		D_18_F23	**340**	*Cladosporium* sp.	100 %
		D_18_F24	341	*Stagonospora* sp.	99 %
		D_18_F25	342	*Cladosporium* sp.	100 %
		D_18_F26	**343**	*Epicoccum nigrum*	100 %
		D_18_F27	344	*Epicoccum nigrum*	100 %
		D_18_F28	345	*Stagonospora* sp.	99 %
		D_18_F29	346	*Leptobacillium leptobactrum*	100 %
		D_18_F30	**347**	*Leptobacillium leptobactrum*	100 %
		D_18_F31	**348**	*Phaeosphaeria halima*	99 %
		D_18_F32	**349**	*Geosmithia* sp.	99 %
		D_18_F33	350	*Acremonium potronii*	99 %
		D_18_F34	351	*Alternaria* sp.	100 %
		D_18_F35	352	*Stagonospora* sp.	99 %
	19	D_19_F1	353	*Botrytis* sp.	100 %
		D_19_F2	354	*Stagonospora* sp.	99 %
		D_19_F3	355	*Stagonospora* sp.	99 %
		D_19_F4	356	*Stagonospora* sp.	99 %
		D_19_F5	357	*Cladosporium* sp.	100 %
		D_19_F6	358	*Stagonospora* sp.	99 %
		D_19_F7	**359**	*Emericellopsis pallida*	100 %

		D_19_F8	**360**	*Cladosporium* sp.	100 %
		D_19_F9	361	*Cladosporium* sp.	100 %
		D_19_F10	**362**	*Stagonospora* sp.	99 %
		D_19_F11	363	*Stagonospora* sp.	99 %
		D_19_F12	364	*Leptobacillium leptobactrum*	100 %
		D_19_F13	365	*Parasarocladium radiatum*	97 %
		D_19_F14	366	*Stagonospora* sp.	99 %
		D_19_F15	367	*Stagonospora* sp.	99 %
		D_19_F16	368	*Cladosporium* sp.	100 %
		D_19_F18	369	*Phaeosphaeria halima*	99 %
		D_19_F19	**370**	*Phaeosphaeria spartinicola*	99 %
		D_19_F20	371	*Leptobacillium leptobactrum*	100 %
		D_19_F21	372	*Botrytis* sp.	100 %
E	20	E_20_F1	373	*Cladosporium* sp.	100 %
		E_20_F2	374	*Leptobacillium leptobactrum*	100 %
		E_20_F3	375	*Exophiala oligosperma*	100 %
		E_20_F4	376	*Exophiala oligosperma*	100 %
		E_20_F5	377	*Exophiala oligosperma*	100 %
		E_20_F6	**378**	*Exophiala oligosperma*	100 %
		E_20_F7	**379**	*Penicillium* sp.	99 %
		E_20_F9	380	*Exophiala oligosperma*	100 %
		E_20_F10	**381**	*Penicillium* sp.	100 %
		E_20_F12	**382**	*Exophiala oligosperma*	100 %
	21	E_21_F1	383	*Aspergillus* sp.	100 %
		E_21_F2	384	*Penicillium* sp.	100 %
		E_21_F3	**385**	*Arthrinium* sp.	99 %
		E_21_F5	387	*Cladosporium* sp.	99 %
		E_21_F6	388	*Acremonium potronii*	99 %
		E_21_F7	**389**	*Acremonium potronii*	99 %
		E_21_F8	**390**	*Parasarocladium radiatum*	98 %
		E_21_F9	**391**	*Parasarocladium radiatum*	98 %
		E_21_F10	**392**	*Penicillium* sp.	99 %
		E_21_F11	**393**	*Trichoderma asperellum*	100 %
		E_21_F12	394	*Cladosporium* sp.	99 %
		E_21_F13	395	*Prasarocladium radiatum*	96 %
		E_21_F14	**396**	*Sarocladium subulatum*	97 %
		E_21_F15	**397**	*Parasarocladium radiatum*	95 %
		E_21_F16	398	*Acremonium potronii*	99 %
		E_21_F17	**399**	*Botrytis* sp.	100 %
		E_21_F18	**400**	*Cladosporium* sp.	99 %
		E_21_F19	**401**	*Acremonium potronii*	99 %
		E_21_F20	402	*Botrytis* sp.	100 %
		E_21_F21	403	*Botrytis* sp.	100 %
		E_21_F22	**404**	*Penicillium* sp.	100 %
	22	E_22_F1	405	*Alternaria alternata*	100 %
		E_22_F2	406	*Alternaria* sp.	100 %

	E_22_F3	407	*Cladosporium* sp.	99 %
	E_22_F4	**408**	*Aspergillus* sp.	100 %
	E_22_F5	**409**	*Parasarocladium radiatum*	96 %
	E_22_F6	**410**	*Trichoderma* sp.	100 %
	E_22_F7	411	*Trichoderma* sp.	100 %
	E_22_F8	412	*Trichoderma* sp.	100 %
	E_22_F9	413	*Parasarocladium radiatum*	96 %
	E_22_F10	**414**	*Acremonium potronii*	99 %
	E_22_F11	415	*Cladosporium* sp.	100 %
	E_22_F12	416	*Alternaria alternata*	100 %
	E_22_F13	417	*Parasarocladium radiatum*	96 %
	E_22_F14	**418**	*Parasarocladium radiatum*	96 %
	E_22_F15	**419**	*Parasarocladium radiatum*	96 %
	E_22_F16	**420**	*Parasarocladium radiatum*	96 %
	E_22_F17	421	*Prasarocladium radiatum*	96 %
	E_22_F18	**422**	*Cladosporium* sp.	100 %
	E_22_F19	**423**	*Cladosporium* sp.	100 %
	E_22_F20	424	*Parasarocladium radiatum*	96 %
	E_22_F21	425	*Cladosporium cladosporioides*	100 %
	E_22_F23	426	*Parasarocladium radiatum*	98 %
	E_22_F24	427	*Arthrinium arundinis*	99 %
	E_22_F25	**428**	*Arthrinium arundinis*	99 %
	E_22_F26	**429**	*Cladosporium* sp.	99 %
	E_22_F27	**430**	*Acremonium potronii*	99 %
	E_22_F28	431	*Cladosporium* sp.	99 %
	E_22_F29	432	*Cladosporium* sp.	99 %
	E_22_F30	433	*Cladosporium* sp.	99 %
	E_22_F31	434	*Cladosporium* sp.	99 %
	E_22_F32	**435**	*Acremonium potronii*	99 %
	E_22_F33	436	*Cladosporium cladosporioides*	100 %
	E_22_F34	437	*Cladosporium* sp.	99 %
	E_22_F35	438	*Cladosporium* sp.	100 %
	E_22_F36	439	*Cladosporium* sp.	100 %
	E_22_F37	**440**	*Aspergillus* sp.	99 %
	E_22_F38	**441**	*Trichoderma* sp.	100 %
	E_22_F39	**442**	*Acremonium potronii*	97 %
	E_22_F40	443	*Emericellopsis pallida*	100 %
	E_22_F43	445	*Trichoderma* sp.	100 %
	E_23_F1	446	*Cladosporium cladosporioides*	99 %
	E_23_F2	**447**	*Acremonium potronii*	99 %
	E_23_F3	448	*Acremonium potronii*	99 %
	E_23_F4	449	*Parasarocladium radiatum*	96 %
23	E_23_F5	450	*Cladosporium* sp.	100 %
	E_23_F6	451	*Cladosporium* sp.	100 %
	E_23_F7	452	*Parasarocladium radiatum*	98 %
	E_23_F8	453	*Cladosporium* sp.	100 %
	E_23_F9	454	*Cladosporium* sp.	100 %

		E_23_F10	**455**	*Cladosporium* sp.	99 %
		E_23_F11	456	*Acremonium potronii*	99 %
		E_23_F12	**457**	*Parasarocladium radiatum*	98 %
		E_23_F13	458	*Cladosporium* sp.	99 %
		E_23_F14	**459**	*Emericellopsis maritima*	99 %
		E_23_F15	**460**	*Acremonium potronii*	99 %
		E_23_F16	**461**	*Acremonium potronii*	99 %
		E_23_F17	462	*Cladosporium* sp.	99 %
		E_23_F18	**463**	*Penicillium crustosum*	100 %
		E_23_F19	464	*Acremonium potronii*	99 %
		E_23_F20	**465**	*Acremonium potronii*	99 %
		E_23_F21	466	*Acremonium potronii*	99 %
		E_23_F22	467	*Acremonium potronii*	99 %
		E_23_F23	**468**	*Parasarocladium radiatum*	96 %
		E_23_F24	**469**	*Acremonium potronii*	99 %
		E_23_F25	**470**	Gen. nov.	-
		E_23_F26	**471**	*Penicillium* sp.	100 %
F	24	F_24_F1	**472**	*Stemphylium vesicarium*	99 %
		F_24_F2	473	*Cladosporium* sp.	100 %
		F_24_F3	474	*Stemphylium globuliferum*	100 %
		F_24_F4	475	*Cladosporium* sp.	100 %
		F_24_F5	476	*Botryosporium longibrachiatum*	99 %
		F_24_F6	477	*Stemphylium vesicarium*	99 %
		F_24_F7	478	*Cladosporium* sp.	100 %
		F_24_F9	**479**	*Cladosporium* sp.	100 %
		F_24_F10	480	*Cladosporium* sp.	100 %
G	25	G_25_F1	**481**	*Penicillium* sp.	99 %
		G_25_F2	482	*Penicillium brocae*	100 %
		G_25_F3	**483**	*Cladosporium* sp.	100 %
		G_25_F4	484	*Cladosporium* sp.	100 %
		G_25_F5	485	*Leptobacillium leptobactrum*	100 %
		G_25_F6	486	*Penicillium brevicompactum*	99 %
		G_25_F11	**488**	*Cladosporium* sp.	100 %
		G_25_F12	**489**	*Meira* sp.	100 %
	26	G_26_F2	490	*Exophiala oligosperma*	100 %
		G_26_F3	491	*Exophiala oligosperma*	100 %
		G_26_F4	**492**	*Exophiala oligosperma*	99 %
		G_26_F5	**493**	*Cladosporium sp*	100 %
		G_26_F6	494	*Exophiala oligosperma*	100 %
		G_26_F7	495	*Cladosporium* sp.	100 %
		G_26_F8	496	*Cladosporium* sp.	100 %
		G_26_F9	**497**	*Aspergillus* sp.	97 %

The sequencing of ITS has a reliable discriminatory power inter- and intraspecific in the fungal species (Schoch *et al*., 2012). Internal Transcribed Spacer has been indicated as the "primary fungal

barcode marker" due its higher discrimination power. In addition, ITS has superior PCR amplification success rate than other ribosomal markers (such as the large subunit and the small subunit of the nuclear ribosomes) (Schoch *et al*., 2012).

The 213 representative isolates allowed the genus/specie identification of the remaining fungal individuals (Table 18). The overall diversity of the fungi isolates resulted in 486 individuals distributed by two subkingdoms, three phyla, seven classes, 11 orders, more than 24 families (due the polyphyletic genera) and 33 different genera (Table 19). It is important to highlight the existence of some genera which can be included in more than one family, due their polyphyletic property as happens with *Acremonium* and *Emericellopsis* species. These two genera have species which can be introduce in distinct families.

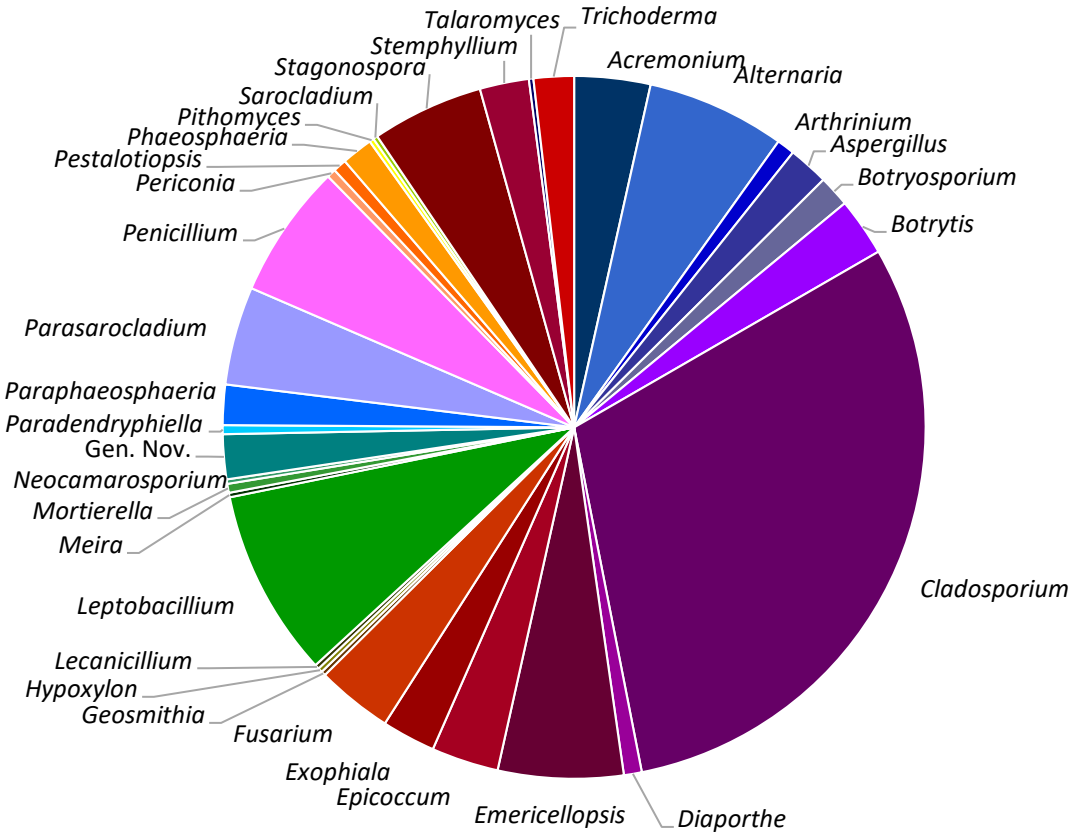

FIGURE 2 | Overall fungal diversity distributed by genera.

The most abundant genus was *Cladosporium* (≈30.25 %), followed by *Leptobacillium* (≈8.64 %), *Alternaria* (≈ 6.38 %) and *Penicillium* (≈6.17 %), all of them included in the phylum Ascomycota. Together, the ascomycetes represented 99.59 % of the overall diversity (Figure 2). Within Ascomycota, the class Dothideomycetes was the most abundant (53.93 %), followed by

Sordariomycetes (30.99 %), Eurotiomycetes (10.74 %) and Leotiomycetes (2.69 %). Cladosporiaceae was the most abundant family, composing 30.25 % of the overall diversity and 56.32 % within the Dothideomycetes group. Only one genus was observed in this family: *Cladosporium*. The second most abundant family, Pleosporaceae, represented 16.86 % of the Dothideomycetes class, and it was populated by isolates belonging to the genera *Alternaria*, *Paradendryphiella* and *Stemphylium*. The Sordariomycetes class was also well represented, mainly due to the high occurrence of the genus *Leptobacillium* (family Cordycipitaceae), *Emericellopsis* and *Acremonium* species (polyphyletic groups in the Hypocreales order) and *Parasarocladium* (included in family Sarocladiaceae) which corresponded to 28 %, 18.67 %, 11.33 % and 14.67 % of genera abundance inside the class, respectively. In the class Eurotiomycetes, two genera were highlighted with 57.69 % and 23.08 %, *Penicillium* (belonging to Aspergillaceae) and *Exophiala* (in the Herpotrichiellaceae family), respectively. Classes Sordariomycetes and Eurotiomycetes have been reported in marine environments in works aiming the endophytic mycobiota of a Mediterranean green alga (Gnavi *et al*., 2017), supporting our data. The class Leotiomycetes, which was the least abundant class, was entirely populated by *Botrytis* species in our collection. Unexpectable, the information about *Botrytis* species associated with marine environment is very scarce. The only reference found that links this genus to marine environment is registered in a compiled list of 2001, organized by Nicholas Clipson and colleagues about the European marine fungi community, where the species *Botrytis cinerea* was included (Costello *et al*., 2001). In this work, *Botrytis* spp. was detected spread by various algae samples from different sampling sites and represents 2.67 % of the overall diversity (13 isolates).

The less represented genera are composed by single species (fill only 0.21 % of the overall diversity for each genera) belong to *Geosmithia, Hypoxylon, Lecanicillium, Leptosphaerulina, Meira, Neocamarosporium, Sarocladium* and *Talaromyces.*

TABLE 19 | Phylogenetic organization of the fungal isolates found in this work (MycoBank information, May 2019; * - Chen *et al*., 2017).

Phylum	Sub-phylum	Class	Subclass	Order	Family	Genus	Isolates
Dikarya							
Ascomycota	Pezizomycotina	Dothideomycetes	Dothideomycetidae	Capnodiales	Cladosporiaceae	*Cladosporium*	147
			Pleosporomycetidae	Pleosporales	Astrosphaeriellaceae	*Pithomyces*	1
					Didymellaceae	*Epicoccum*	15
					Didymosphaeriaceae	Putative new genus	10
						Paraphaeosphaeria	9
					Massarinaceae	*Stagonospora*	25
					Neocamarosporiaceae	*Neocamarosporium*	1
					Phaeosphaeriaceae	*Phaeosphaeria*	7
					Periconiaceae	*Periconia*	2
					Pleosporaceae	*Alternaria*	31
						Paradendryphiella	2
						Stemphylium	11
		Eurotiomycetes	Chaetothyriomycetidae	Chaetothyriales	Herpotrichiellaceae	*Exophiala*	12
			Eurotiomycetidae	Eurotiales	Aspergillaceae	*Aspergillus*	9
						Penicillium	30
					Trichocomaceae	*Talaromyces*	1
		Leotiomycetes	Leotiomycetidae	Helotiales	Sclerotiniaceae	*Botrytis*	13
		Sordariomycetes	Hypocreomycetidae	Hypocreales	Polyphyletic group	*Acremonium*	17
						Emericellopsis	28
					Cordycipitaceae	*Leptobacillium*	42
						Lecanicillium	1*

TABLE 19 | Phylogenetic organization of the fungal isolates found in this work (continued).

Ascomycota	Pezizomycotina	Sordariomycetes	Hypocreomycetidae	Hypocreales	Hypocreaceae	*Trichoderma*	9
					Nectriaceae	*Fusarium*	17
					Sarocladiaceae	*Parasarocladium*	22
						Sarocladium	1
					Incertae sedis	*Geosmithia*	1
			Sordariomycetidae	Diaporthales	Diaporthaceae	*Diaporthe*	4
			Xylariomycetidae	Xylariales	Apiosporaceae	*Arthrinium*	4
					Hypoxylaceae	*Hypoxylon*	1
				Amphisphaeriales	Sporocadaceae	*Pestalotiopsis*	3
	Ascomycota *incertae sedis*					*Botryosporium*	7
Basidiomycota	Ustilaginomycotina	Exobasidiomycetes	Exobasidiomycetidae	Exobasidiales	Brachybasidiaceae	*Meira*	1
Mucoromyceta							
Mortierellomycota	Mortierellomycotina	Mortierellomycetes	-	Mortierellales	Mortierellaceae	*Mortierella*	2

Apart from the detection of isolates belonging to Dikarya (Ascomycota and Basidiomycota fungi), it was detected two isolates belonging to a genus included in a different subkingdom, Mucormycota. These two individuals (0.41 % of diversity) belong to the genus *Mortierella* (Table 19). Usually, *Mortierella* species are isolated from soil (Hyde *et al*., 2017), decomposing matter (Hyde *et al*., 2016), but some species were already associated with marine environment (Costello *et al*., 2001) and Antarctic red and green algae (Ogaki *et al*., 2019). This survey is the first report of *Mortierella* species associated to *Fucus spiralis*, in Portugal. However, our data are not strong enough to say that this genus is adapted to algae hosts. It was found only two isolates in the same site at the same host, without previous references which establish an association of *Mortierella* species with *Fucus spiralis*. Similarly, Du *et al*. (2019) presented a work where *Mortierella* species exhibited a better adaptation to the absence of nutrients in the medium when in the presence of the alga *Nannochloropsis oceanica*. Although being isolated from soil substrate, *Mortierella elongata* was the only fungus capable of integrate the algal cells in their hyphae, promoting the nitrogen and carbon shifts between both organisms (Du *et al*., 2019).

In Table 20, a relation between families is established in order to include the less represented genera in the analysis. Table 20 also presents the distribution of each family and the proportion of the genera inside of the respective group. This analysis allowed us to observe that the majority of the families are represented by a single genus. Of a total of more than 23 families, only 5 families included more than one genus, namely Didymosphaeriaceae, which included a putative new genus (Gen. nov., to be characterised and taxonomically described in a future study) and *Paraphaeosphaeria*; Aspergillaceae, which comprised two related genera, *Penicillium* and *Aspergillus*; Sarocladiaceae, a recently introduced family, which includes *Sarocladium* and *Parasarocladium* isolates; Cordycipitaceae which incorporates the genera *Leptobacillium* and *Lecanicillium*; and family Pleosporaceae, with a considerable presence, including species from the genera *Alternaria*, *Stemphylium* and, less extensively, the marine genus *Paradendryphiella* (previously, identified as *Dendryphiella* species) (Woudenberg *et al*., 2013).

The absence of species belonging to Halosphaeriaceae and Lulworthiaceae families is noteworthy, since it contradicts the results obtained by PCR-DGGE approach in previous studies using algae hosts belonging to *Fucus* genus (Raghukumar, 2012). It is important to highlight that these fungal families were detected by independent cultivation methods, while this survey was performed through dependent cultivation methods (Zuccaro *et al*., 2008). Such differences like the variability of the conditions of fungi isolation e.g. the composition of media used, can lead to the

recovery of distinct elements of the fungal community (Fan *et al*., 2019). In addition, the growth time of the fungi also can influence the results obtained. It is also important to highlight that this survey only includes the isolates which appeared after five days of cultivation at 25 ^{o}C (in the dark).

TABLE 20 | Relative abundance (%) of the families and respective abundance of the genera in the respective family.

Family	Total of isolates	Relative abundance (%)	Genera	Isolates	Abundance in the family (%)
Apiosporaceae	4	0.82	*Arthrinium*	4	100
Aspergillaceae	39	8.02	*Aspergillus*	9	23,08
			Penicillium	30	76,92
Astrosphaeriellaceae	1	0.21	*Pithomyces*	1	100
Brachybasidiaceae	1	0.21	*Meira*	1	100
Cladosporiaceae	147	30.25	*Cladosporium*	147	100
Cordycipitaceae	43	8.85	*Leptobacillium*	42	97.67
			Lecanicillium	1	2.33
Diaporthaceae	4	0.82	*Diaporthe*	4	100
Didymellaceae	15	3.09	*Epicoccum*	15	100
Didymosphaeriaceae	19	3.91	*Paraphaeosphaeria*	9	47,37
			Gen. nov.	10	52,63
Herpotrichiellaceae	12	2.47	*Exophiala*	12	100
Hypocreaceae	9	1.85	*Trichoderma*	9	100
Hypoxylaceae	1	0.21	*Hypoxylon*	1	100
Incertae sedis	1	0.21	*Geosmithia*	1	100
Incertae sedis	7	1.44	*Botryosporium*	7	100
Massarinaceae	25	5.14	*Stagonospora*	25	100
Mortierellaceae	2	0.41	*Mortierella*	2	100
Nectriaceae	17	3.5	*Fusarium*	17	100
Neocamarosporiaceae	1	0.21	*Neocamarosporium*	1	100
Periconiaceae	2	0.41	*Periconia*	2	100
Phaeosphaeriaceae	7	1.44	*Phaeosphaeria*	7	100
Pleosporaceae	44	9.05	*Paradendryphiella*	2	4,55
			Stemphylium	11	25
			Alternaria	31	70,45
Polyphyletic group	17	3.50	*Acremonium*	17	100
Polyphyletic group	28	5.76	*Emericellopsis*	28	100
Sarocladiaceae	23	4.73	*Sarocladium*	1	4,35
			Parasarocladium	22	95,65
Sclerotiniaceae	13	2.67	*Botrytis*	13	100
Sporocadaceae	3	0.62	*Pestalotiopsis*	3	100
Trichocomaceae	1	0.21	*Talaromyces*	1	100

At the order level, Pleosporales was the most diverse order found, exhibiting a distribution of the isolates by 8 distinct families and 12 genera. Although this group comprises 23.46 % of the whole isolates, it is not the most abundant order. Capnodiales (which it is entirely composed by *Cladosporium* species) and Hypocreales (mainly composed by *Leptobacillium* isolates) are the two orders most representatives, corresponding to 30.25 % and 28.40 % of the fungal isolates collection, respectively. The following orders are composed by low amounts of isolates, exhibiting a big difference with the most representative fungal groups. Eurotiales (8.23 %) is the fourth most abundant order and, in addition with the Chaetothyriales (2.47 %), both make part of the Eurotiomycetes class, commonly found in marine environments in other similar works (Gnavi *et al*., 2017).

Representing 53.93 % of Ascomycota, Dothideomycetes was the most prevailing class of fungi found in this work, being divided by *Cladosporium*, *Epicoccum*, *Paraphaeosphaeria*, Gen. nov., *Stagonospora*, *Neocamarosporiunm*, *Periconia*, *Alternaria*, *Paradendryphiella* and *Stemphylium.* All these genera belong to Pleosporales order, except *Cladosporium* genus, which is included in Capnodiales order. It makes sense the high presence of this group in Ria de Aveiro because it is an environment characterized as intertidal with mangrove zones (Suetrong *et al*., 2009), representing a favourable environment for the marine Dothideomycetes. This class, in addition with an order belonging to Sordariomycetes class, Hypocreales, already had been referred as endophytes in algae hosts (*Fucus* sp.) in previous studies (Raghukumar, 2012; Zuccaro *et al.*, 2008). So, it is not a surprise that they possess a large presence in the fungi algicolous assemblages of algae from Ria de Aveiro. According to the data obtained, the Hypocreales order is well represented through the genera *Acremonium*, *Leptobacillium*, *Trichoderma*, *Fusarium*, *Parasarocladium* and *Emericellopsis*.

In addition to the genera indicated before, other fungal groups can be highlighted in the present survey, such as the three genera related belonging to the Eurotiales order (*Penicillium*, *Talaromyces* and *Aspergillus*); *Exophiala* species from Chaetothyriales; *Botrytis*, *Diaporthe*, *Arthrinium*, *Hypoxylon* and *Pestalotiopsis* belonging to Sordariomycetes class; and one "unclassified" Ascomycota fungus, the *Botryosporium* isolate.

Our data reinforced the phylum Ascomycota as the most common endophytes in algae-inhabiting fungi (Gnavi *et al*., 2017). The very rare presence of basidiomycetes in this study can be supported by the inability of this fungal group to colonize the algal cellulose present in the thalli

(Gnavi *et al*., 2017). In fact, *Meira*, belonging to the family Brachybasidiaceae, was the only basidiomycete found.

4.3. COMMUNITY STRUCTURE AND MULTIVARIATE ANALYSES

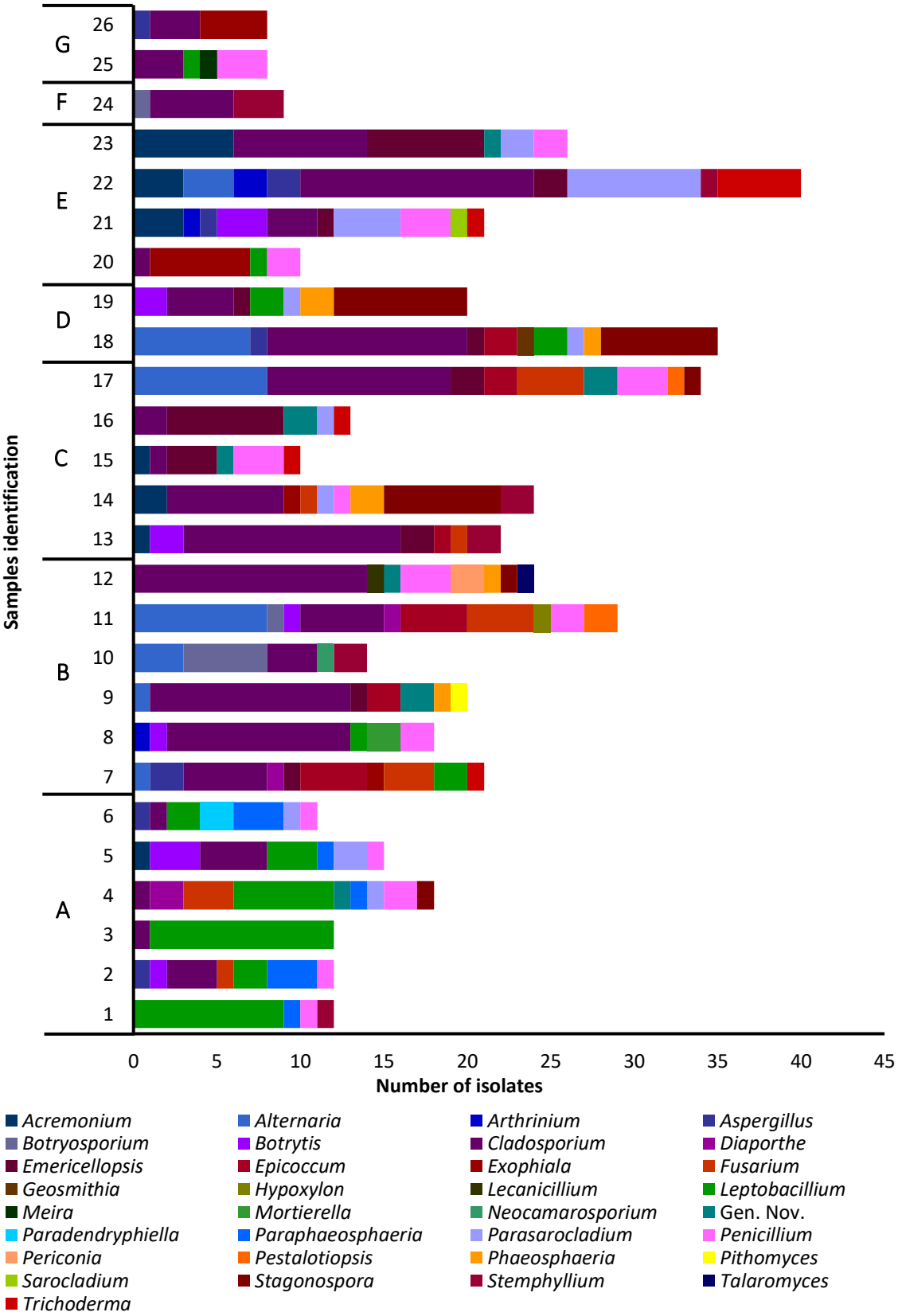

FIGURE 3 | Fungi community distributed by algae hosts. **A-G** refers to the sampling sites.

Figure 3 gives a general scenario about the distribution of fungal genera according to algae samples and reveals the absence of a common fungal genus across all seaweeds collected. *Cladosporium* was the most widespread genus by across all hosts, being absent in just one alga collected from site A, (sample 1: *Ulva* sp.). Although *Cladosporium* is a strongly represented genus in our isolates collection, its distribution did not occur equally for all the hosts and/or sites. *Penicillium* was the second most widespread genus, being observed in 15 algae samples and 11 different hosts. This genus was abundant in *Gracilaria* sp. (site C) and *Codium* sp. (site G). *Leptobacillium* was the third genus most disseminated, being mostly presented in the hosts collected from the site A, where it is also dominant along the fungi community of the hosts collected from this site. In addition, the order Hypocreales was also mainly represented by two distinct genera: *Emericellopsis* in *Ulva* sp. and *Gracilaria* sp. (both collected from site C) and *Parasarocladium* isolated from an unidentified red alga (site E). There is a solid presence of genera belonging to order Pleosporales, including *Alternaria* species (very abundant in *Fucus* hosts collected from site B) and *Stagonospora* in *Cladophora* sp. (site D). Species belonging to *Exophiala* genus were well represented in *Ceramium* algae (site E) and *Scinaia* sp. (site G). One sample collected from site B, identified as *Fucus* sp., showed high abundance of *Botryosporium* sp., an "*Ascomycete incertae sedis*". This last genus has been associated with mangrove in another studies (Zhao & Zhou, 2005).

Samples were also analysed for their richness (as number of species), diversity (using Shannon's *H´* diversity and Simpson's diversity) and evenness (Pielou) (Table 21). The Pielou's evenness calculates the relative abundance of the different species within a community (in this case, in the samples), and measures the "ratio of the observed diversity to the maximum possible in a collection having the same number of species". This value is obtained using a formula derived from Shannon's diversity index and it is a complement for the diversity analysis (Motwani *et al*., 2014; Pielou, 1966).

TABLE 21 | Richness, diversity and evenness indices from algae samples (sample = alga sample). Red alga 1 – algae samples belonging to the same genus. *- unknown algae genus.

Site	Sample	Algae Host	Richness (R)	Shannon diversity index (H)	Simpson diversity index (S)	Evenness Pielou index (J)
A	1	Green alga: *Ulva* sp.	4	0.84	0.417	0.604
	2	Red alga 1*	7	1.82	0.819	0.935
	3	Green alga: *Ulva* sp.	2	0.29	0.153	0.414
	4	Red alga 1*	9	1.96	0.821	0.89
	5	Brown alga: *Fucus* sp.	7	1.81	0.818	0.928
	6	Brown alga: *Fucus* sp.	7	1.85	0.826	0.949
B	7	Brown alga: *Fucus spiralis*	10	2.11	0.857	0.916
	8	Brown alga: *Fucus spiralis*	6	1.27	0.593	0.709
	9	Green alga: *Enteromorpha* sp.	7	1.37	0.61	0.702
	10	Brown alga: *Fucus* sp.	5	1.49	0.755	0.929
	11	Brown alga: *Fucus* sp.	10	2.04	0.842	0.885
	12	Brown alga: *Fucus* sp.	8	1.44	0.628	0.694
C	13	Filamentous green alga*	7	1.39	0.62	0.712
	14	Brown alga: *Fucus* sp.	9	1.87	0.802	0.851
	15	Red alga: *Gracilaria gracilis*	6	1.64	0.78	0.917
	16	Green alga: *Ulva* sp.	5	1.3	0.651	0.81
	17	Green alga: *Ulva* sp.	9	1.88	0.806	0.855
D	18	Green alga: *Cladophora* sp.	10	1.85	0.792	0.802
	19	Green alga: *Cladophora* sp.	7	1.68	0.765	0.863
E	20	Red alga: *Ceramium* sp.	4	1.09	0.58	0.785
	21	Red alga*	10	2.15	0.871	0.935
	22	Green alga: *Ulva* sp.	9	1.88	0.802	0.855
	23	Green alga: *Enteromorpha intestinalis*	6	1.57	0.766	0.879
F	24	Brown alga: *Fucus* sp.	3	0.94	0.568	0.853
G	25	Green alga: *Codium* sp.	4	1.26	0.688	0.906
	26	Red alga: *Scinaia* sp.	3	0.97	0.594	0.887

The highest richness value observed, in the samples represented in Table 21, was 10 in four samples: two brown algae (2 samples belonging to *Fucus* sp.), one red alga (unknown genus) and one green algae (*Cladophora* sp.). In opposition to these results, the lowest richness value was observed for *Ulva* sp., collected in site A, where only two unique species were isolated.

The measures of both diversity indices (Shannon and Simpson) follow similar patterns to those of the richness, although there are slight differences between samples which have the same number of unique species. The sample with the highest value of Shannon diversity index (H) is a red

alga collected from site E (H = 2.15), while the lowest diversity value corresponded to an *Ulva* sp., collected in site A (H = 0.29). Shannon´s H diversity values higher than 2.0 were found in red and brown algae, suggesting that these hosts are the "richest" samples possessing more different fungal species than the remaining hosts. Regarding the values obtained by Simpson diversity index (S), it is possible to conclude that there is in concordance with the results obtained in Shannon diversity index. The highest value reached was 0.871 from a red alga (site E), and the lowest value was also verified in site A, in *Ulva* sp. (S = 0.153). Grouping host by algae groups, the red and brown algae are the samples with the highest diversity values for the both indices. This situation can be explained by the shorter life cycle of the green algae in comparison with red and brown algal species, avoiding the growth and development of slowest fungi in green algae. In addition, the slow growth of typical endophytes of these hosts can be another reason for the lower diversity verified in green algae (Raghukumar, 2012).

Analysis of Pielou´s evenness (J) allowed us to determine that the most even sample in species distribution was a brown alga from site A (J = 0.949), while the most diverse only had 0.89 of evenness. This means that the most diverse sample exhibits more differences between the relative abundance of each fungi in the community sheltered. Once again, red and brown algae are the hosts with the highest values, presenting a more even distribution of their fungal species. Higher values of evenness indicate an equative distribution of fungal community in the algae tissues. It is important to highlight the results obtained in site E, where the highest value of evenness and richness indices correspond exactly to the same sample (a red alga). However, this situation only allows us to affirm that this sample presents a high fungal diversity, and a uniform distribution of each species in their community. On the other hand, two green algae are the hosts with more discrepancies between the fungi abundance that they shelter (*Ulva* sp. and a filamentous green algae). Site G presents a well distributed fungi community (J = 0.90), followed by E (0.86), F (0.85), D (0.83) and C (0.83). The lowest value of evenness is from site A (0.79).

The most important factors implicated in the fungi assemblage community of the hosts are a controversial topic between mycologists. Physico-chemical conditions such as temperature, salinity and pH are environmental influencers for the fungi assemblage. However, when considering a "host" and "fungi community", more variables can be suggested as influencers beyond the environmental conditions (Abdel-Gawad *et al*., 2014). In spite of the importance given to geographical localization, as a determinant influencer of the fungi community composition, it is

important do not depreciate studies where the host genera are present as an influencer with equivalent importance (Abdel-Gawad *et al*., 2014; Harvey & Goff, 2010; Ogaki *et al*., 2019).

The influence of the host algae in richness, Shannon's H diversity, Simpson's dominance index and Pielou's evenness of the samples was then tested, and no significant differences were found. These results indicate that the host algae are not an influencer in the fungal diversity of the algicolous communities in this study.

In a posterior analysis, after grouping algal hosts in green, brown and red algae, significant differences were detected between these three groups (Kruskal-Wallis: p-value = 0.0417; p-value < 0.05). The post-hoc Nemenyi-Test revealed significant differences in Pielou's evenness between the red and green algae samples (p-value < 0.05) (Table 22).

TABLE 22 | Results of the p-values obtained from Nemenyi-Test, exhibiting the groups with significative differences between algae groups (green, red and brown algae).

Pairwise comparisons	Brown	Green
Green	0.216	-
Red	0.652	0.044

This result suggests that the species distribution does not present the same fungi uniformity level between these two macroalgae divisions. Considering the data present in Table 21, it can be observed that the Pielou´s evenness values are higher for red than for green algae. This result indicates a more even distribution of the fungal species in red algae, while the fungi species in green algae present a situation of dominance of some or several species.

In Figure 4, when the fungi are distributed according to macroalgae group, it is possible to observe that the *Cladosporium* abundance in green algae is dominant. Comparing *Cladosporium* abundance with the quantity of other taxonomic groups present in green algae, a noteworthy discrepancy is verified. This difference can account for the lowest Pielou´s evenness index value verified in green algae. On the other hand, the community fungi of red algae are more quantitatively distributed, resulting in a higher Pielou´s evenness index values. The significative difference observed between the evenness of these two algae groups can be a result from a non-padronized algae collection (11 samples of green algae vs. 6 samples of red algae).

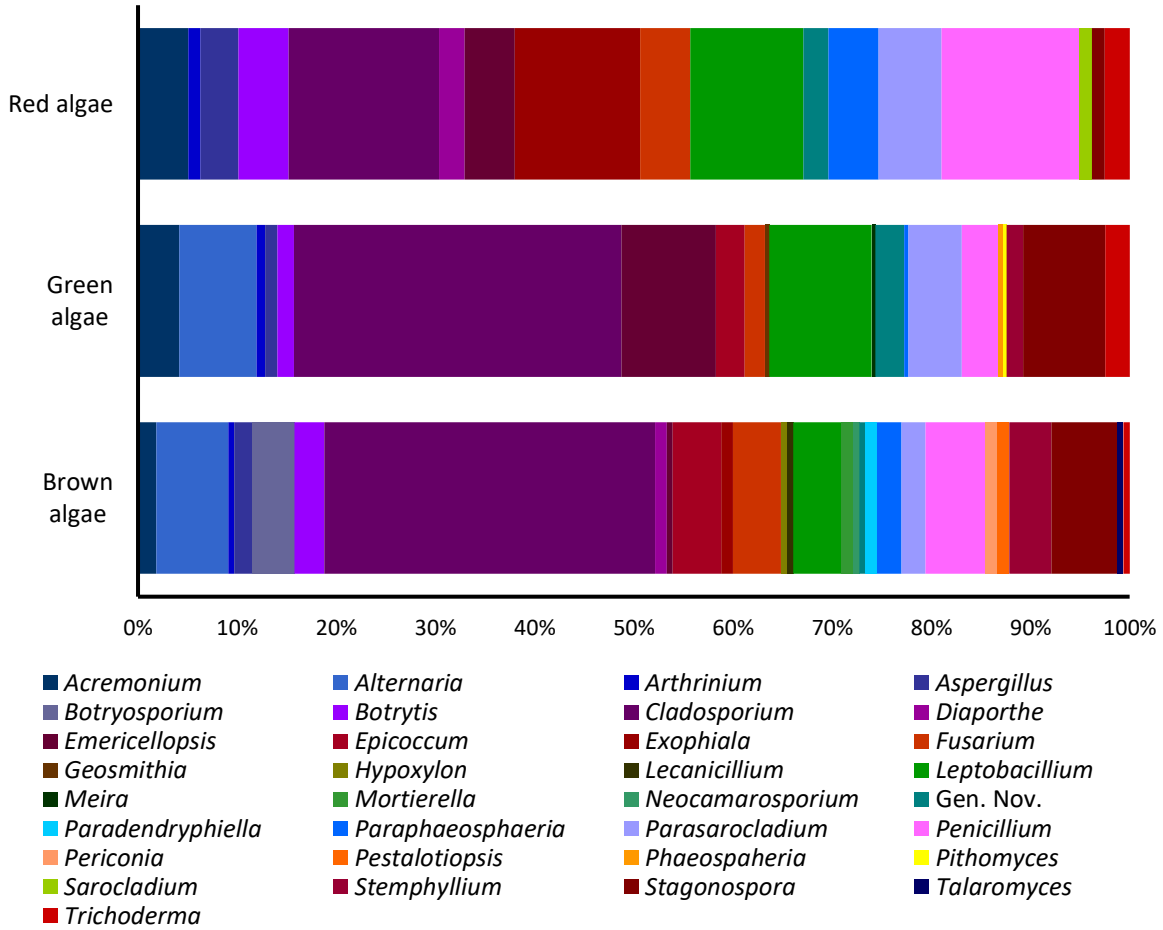

FIGURE 4 | Fungi community distributed by green, red and brown algae.

Multi-level pattern analysis ("multipatt") demonstrated some relations established between fungi genera and algae hosts. A posterior analysis showed an association of *Exophiala* genus with two hosts groups, *Ceramium* sp. and *Scinaia* sp. ("stat" = 0.989, p-value = 0.025), and *Emericellopsis* spp. with green and red algae ("stat" = 0.752, p-value = 0.04). *Exophiala* is a marine algal-derived endophytic, common in red algae, so their association with two genera of the red algae is not a surprise (Li *et al*., 2011). Despite its presence in *Fucus* sp. (brown algae), a higher number of *Exophiala* isolates were found in the red algae, in this work, which is supported by other studies (Li *et al*., 2011). On the other hand, the lowest quantity of isolates identified as "*Emericellopsis* sp." in brown algae is an unexpected result. This situation can be associated with the medium used for fungal isolation from endophytes of *Fucus* sp. A survey by Fan *et al*. (2019) demonstrated that PDA is not always the best suitable method to obtain isolates of *Emericellopsis* species endophytes from *Fucus* spp. Since, *Fucus* spp. are the only genus composing the group of brown algae in this work, the medium can be inappropriate, which accounts for the low quantity of *Emericellopsis* spp. obtained.

Exophiala sp. was the only group associated with some algae genus. All the remaining fungi genera seems not to be related with specific algae hosts. All the statistical results corroborate the independence verified between fungi community's species and respective hosts. This is a situation already proved in other similar work of Harvey & Goff (2010) where they demonstrated that the host is not a determinant factor in the mycobiota assemblage. Although, this situation does not invalidate a slight preference of some fungi group for defined algae host or group (Abdel-Gawad *et al*., 2014; Gnavi *et al*., 2017).

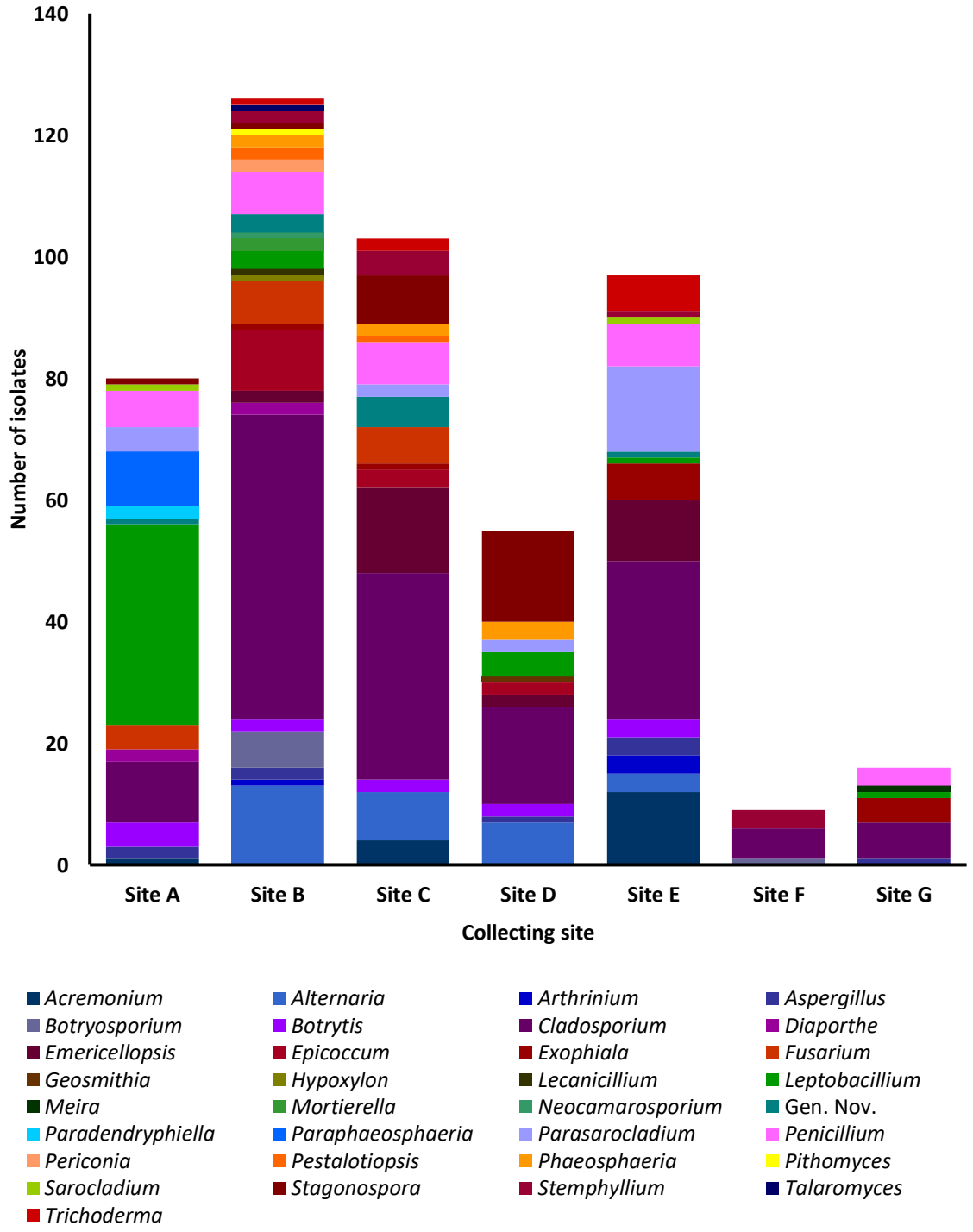

FIGURE 5 | Fungi community of each site of our study.

Figure 5 shows the differences between the mycobiota communities of each sampling site. These differences could be observed at genera presence and/or abundance. Except for sampling site A (where *Leptobacillium* is the dominant genus), *Cladosporium* genus is dominant in the remaining sites though its distribution was not equally distributed by them. The dominance of

Ascomycota species in all these sampling sites can be related with the capacity of these species to inhabit sites with different salinity concentrations, being always widely spread by brackish and salt marsh environments (Mohamed & Martiny, 2011). In site A, the fungal community is clearly dominated by isolates belonging to Sordariomycetes class (*Leptobacillium* and *Parasarocladium*), followed by Dothideomycetes (*Cladosporium*, *Paradendryphiella* and *Stagonospora*) and lastly by isolates from Eurotiomycetes (*Aspergillus* and *Penicillium*).

In sites C and D (central zones of Ria de Aveiro), where the salinity is lower than in B and E (Vaz *et al*., 2005), a different dominant fungal group was observed: *Stagonospora* sp. This facultative marine fungus does not require a specific amount of sodium chloride to grow. It can grow in the presence or absence of salt (Calado *et al*., 2015), which justifies their presence in sites with a low salinity concentration, such as the sites C and D.

In site E, another genus stands out, *Parasarocladium*, a group recently created to accommodate some *Acremonium* species, that previously belonged to *Radiatum* complex (Crous *et al*., 2018; Summerbell *et al*., 2018). Upon cultivation in laboratory, we noticed a better developing and growth in culture medium supplemented with sea salts. This shows a “positive” influence of sodium chloride in the growth of *Parasarocladium* species which supports their occurrence in site E. In this site, the salinity concentration is the least variable between tides, remaining constant and always above 10 (Vaz *et al*., 2005), which can promote the establishment of species with a better growth in the presence of salt, such as *Parasarocladium*.

In site G, the noteworthy fungi are the isolates belonging to the algal-derived endophytic *Exophiala* genus. This site presents a high salinity level since it is located in a marine environment (sea) (Vaz *et al*., 2005). Their association to inner tissues of red algae (*Scinaia* sp.) collected in this site, can be supported by other studies of *Exophiala* in associations to marine algae, *Laurencia similis* (Li *et al*., 2011). In addition, there are records of association of this genus with other algae hosts collected from oceanic coast environments (Li *et al*., 2011). In addition, *Exophiala* sp. can also act as a human pathogenic agent (Listemann & Freiesleben, 1996) responsible for various skin diseases, such as mycoses, and can even infect the human brain (Hoog *et al*., 2003).

Regarding the sites under study, the highest value of Shannon´s and Simpson diversity indices (resulting from the mean of the samples) was in site D (mean = 1.77 and 0.78, respectively), which agree to the richness in this site (R = 8.5). Also, in sites E, B and C, Shannon´s Diversity index presented high values (1.67, 1.62 and 1.62, respectively) as well as Simpson index (0.75, 0.71 and

0.73, respectively). On the other hand, the lowest Shannon and Simpson diversity indices values are verified in site F (mean = 0.94 and 0.57, respectively), followed by site G (1.12 and 0.64, respectively) and A (1.43 and 0.64, respectively). Regarding the evenness of the sites, the site G is the one where the fungal community is more uniform due to the high Pielou index (0.90). On the other hand, the site A has the least even fungal community.

Further statistical analyses were performed in order to understand the influence of the collecting site in fungi community composition (richness, diversity and evenness). To understand these relations of fungal community with the sampling sites, the differences of the indices of the samples across sampling sites were evaluated.

Once again, it is verified the absence of significant differences between the values of richness (Kruskal-Wallis Rank Sum Test: Chi-squared = 8.2577, p-value = 0.2198), Shannon´s diversity Index (Kruskal-Wallis test: p-value = 0.4759), Simpson´s diversity index (Kruskal-Wallis Rank Sum Test: Chi-squared = 3.2399, p-value = 0.7782, p-value > 0.05) and Pielou´s evenness (Kruskal-Wallis: p-value = 0.47) between the samples collected from different sites.

Despite the absence of significant differences between the algae samples from different sampling sites, there are some fungal genera that seem to be more related with some sampling sites (Figure 6).

The distribution of the genera in Figure 6, shows that the genus *Cladosporium* was the only one, of the 33 genera, which was observed in all sampling sites. Twenty-six genera were observed in site B and seven of these were exclusive to this site. This was the case for the genera *Hypoxylon*, *Lecanicillium*, *Mortierella*, *Neocamarosporium*, *Periconia* and *Pithomyces.* Notably, all these groups included only one or two isolates in the overall distribution (Table 19).

Leptobacillium spp., the second most abundant group occurred frequently in sampling site A (78.57 % of the total of *Leptobacillium* isolates was observed in this site), while in local B, D, E and G only have, respectively, 7.14 %, 9.52 % and 2.38 % (site E and G).

Alternaria and *Penicillium*, two of the most abundant genera, were present in four and five sampling sites, respectively. *Penicillium* presents an approximately equitable distribution along the sites while *Alternaria* is predominantly in local B.

Further analysis of the isolates distribution across sampling sites suggested a "site-genus association". Genera as *Paradendryphiella* and *Paraphaeosphaeria* were observed only in sampling site A. *Hypoxylon*, *Lecanicillium*, *Mortierella*, *Neocamarosporium*, *Periconia*, *Pithomyces* and *Talaromyces* are genera that appeared only in sampling site B and *Geosmithia* in site D. The Basidiomycota species belonging to *Meira* appeared only in site G, while the sampling site B sheltered the isolates belonging to the Mortierellomycota phylum, in the Mucoromyceta subkingdom.

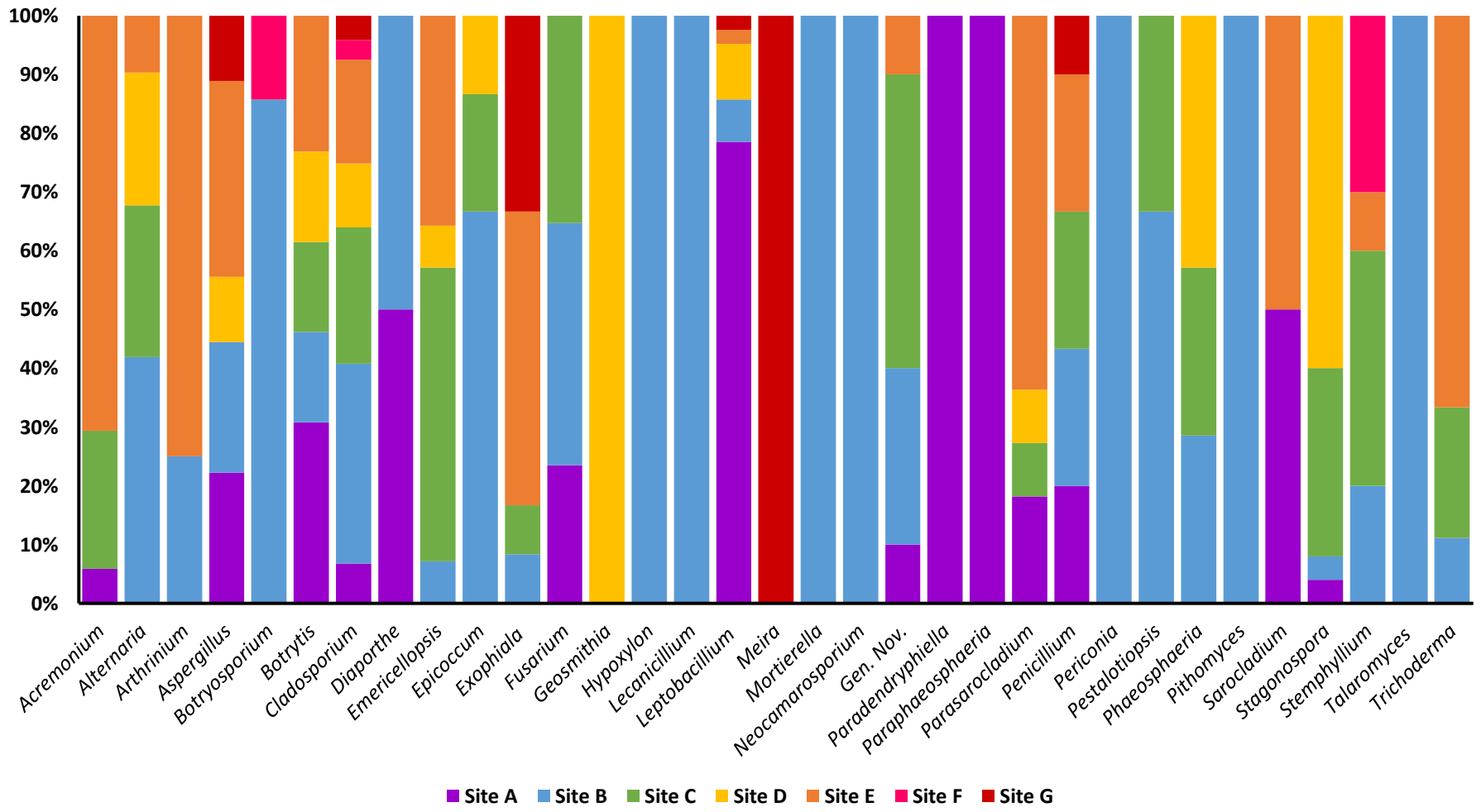

FIGURE 6 | Proportional distribution of each genus by the sites where they were isolated.

Multi-level pattern analysis allowed the detection of some fungal groups associated with some sampling sites. Results from this analysis suggested an association of *Paraphaeosphaeria* spp. and site A ("stat" = 0.913, p-value = 0.025), *Stemphylium* spp. and site F ("stat" = 0.812, p-value = 0.035), while in site D there was an association with *Stagonospora* spp. ("stat" = 0.892, p-value = 0.015) and *Phaeosphaeria* spp. ("stat" = 0.820, p-value = 0.050). Also, *Leptobacillium* spp. was linked to sites A and D ("stat" = 0.926, p-value = 0.005), and *Emericellopsis* spp. was associated with sites C, D and E ("stat" = 0.882, p-value = 0.025). These results are a possible consequence of the unequal distribution of the fungi abundance over the sampling sites. The connection established between the mycoparasite *Paraphaeosphaeria* and *Leptobacillium* with local A are, probably, justified by the proximity of this local to the soil and intense anthropogenic activity. Both fungi groups represent new records in marine environment, due their characteristics as plant endophytes (Nicot *et al*., 2019) and soil inhabitants (Zare & Gams, 2016). However, biotic factors such as wind and rain, or biotic factors like transition organism and humans, can promote the dispersion of the respective fungi spores. In addition, *Leptobacillium* also was associated to local D. Despite their association to this site (using the multi-level pattern analysis), it is important to consider only one alga species was recovered from this site. The abundance of *Leptobacillium* in this site can be considered "accidental", because 4 isolates are not enough to ensure this sampling site as a niche to the fungi genus. *Stagonospora* is a fungal group with a stronger presence in this site, as demonstrated by the statistical analysis (above). This genus is associated with their relative *Phaeosphaeria* (teleomorph of *Stagnospora*), also linked with D site in this survey in the multi-level pattern analysis (Solomon *et al*., 2006). *Phaeosphaeria* is a genus that has the capacity to inhabit a wide type of hosts, such as plants (cereals and grasslike plants) and some marine substrata, including salt marsh (Calado *et al*., 2015; Shoemaker & Babcock, 1989). Some *Phaeosphaeria* spp. are endolichenic and can produce compounds with cytotoxic activity against human tumor cells by photoactivation (Li *et al*., 2012). In addition to *Phaeosphaeria*, also their relative *Stagonospora*, can include marine species (facultative) (Calado *et al*., 2015; WoRMS Editorial Board, 2019). In this work carried out with algae collect from Ria de Aveiro, both genera (*Phaeosphaeria* and *Stagonospora*) were detected in a site with a lower salinity concentration than the remaining sites. It is known that *Stagonospora* has the capability to grow in the absence of salt (Calado *et al*., 2015), and their relative *Phaeosphaeria* also can possess the same ability, explaining their high abundance in site D, where the salinity is low. *Stemphylium*, a genus statistically relevant in site F, situated in the coast near of vegetation, only had three isolates in this site. These isolates can be present in the algae accidently, because this genus has phytopathogenic species included, and the near vegetation can shelter some individuals

belonging to this group, and their spores can reach the marine environment through environment factors (Marin-Felix *et al*., 2019).

Cladosporium genus is the fungal group dominant in the samples collected from the study sites B to G in this survey (Figure 5 and 6). This genus is characterized as "cosmopolitan in distribution" because of its great adaptation ability which allows it to inhabit a wide range of different substrates in distinct environments (Bensch *et al*., 2012). A hypothesis for this successful dissemination could be related with the morphological characteristics of their conidia, which are usually small and branched, allowing for an easier dispersal distribution by distinct ecological niches (Bensch *et al*., 2012). In addition, their dominance in the analysed algae was expected since this genus is well documented as an endophyte in algae hosts (Bensch *et al*., 2012; Thirumalanadhuni & Palempalli, 2018). Species belonging to the genus *Cladosporium* have been reported in brown, red and green algae (Ding *et al*., 2008; Thirumalanadhuni & Palempalli, 2018; Trivedi *et al*., 2015) and have been investigated for their potential to produce antimicrobial, antitumoral and antioxidant substances (Ding *et al*., 2008; Henríquez *et al*., 2013). Also, the considerable presence of *Cladosporium* species in fungal communities where the salinity levels vary under tides, can be explained by their high adaptation to different abiotic conditions in the marine environment (Bensh *et al*., 2012; Raghukumar, 2012). These properties of *Cladosporium* species support their widespread and "dominance" in the sites B, C, D, E, F and G, under different salinities gradient (Bensh *et al*., 2012). This way, our data reinforce *Cladosporium*, as a group with a common presence in a wide range of algae hosts in the marine environment, as well as ecological niches very distinct, which relates to their cosmopolitan character.

The genus *Alternaria* is another group well represented in our samples (Figures 5 and 6). This group belongs to a class widespread and well established in aquatic and marine substrata, the Dothideomycetes (Gnavi *et al*., 2017). Usually, *Alternaria* is a genus "strong" connected with the marine environment but their presence in the algae hosts has not been indicated (Woudenberg *et al*., 2013). On the other hand, the existence of *Alternaria* marine-derived species with antibiotic properties against bacteria has been reported in association with marine corals and sediments (Kim *et al*., 2009; Shaaban *et al*., 2012). As happen to *Alternaria* species, also *Penicillium* is a common endophyte found in similar proportions, in this work (Woudenberg *et al*., 2013; Gonçalves *et al*., 2019[1]). In the present work, *Penicillium* sp. was the most abundant fungi group in *Fucus* sp. (brown algae), red algae and, in less extension, in green algae. *Penicillium* is a large and complex group, sheltering about 1500 different species, organized in subgenera and sections (Visagie *et al*., 2014).

Due the complexity of these species group, it is required a polyphasic approach based on morphological characters, extrolites produced and genetic information (Visagie *et al*., 2014). In addition, it can be found in a wide range of different substrata from terrestrial habitats to marine environments including intertidal habitats, saline waters (including the hypersaline) (Gonçalves *et al*., 2019[1]; Raghukumar, 2008; Visagie *et al*., 2014). This genus is mainly explored due to the potential of its secondary metabolites (such as antibiotic activity), especially the marine-derived species (Edrada *et al*., 2002; Komatsu *et al*., 2000). There are few studies in Portugal that focus on marine fungal communities (Gonçalves *et al*., 2019[1]). However, a recent project has detected a set of *Penicillium* species present in a wide range of substrata, in the Portuguese coast (Gonçalves *et al*., 2019[1]). These results agree with the observations made in the current study.

The second most abundant fungal group in algae from Ria de Aveiro was *Leptobacillium*. This genus was recently introduced in 2016 to include the species previously identified as *Verticillium leptobactrum*. This group is poorly described, and it is frequently associated with decomposing matter in the soil, as saprobic (Zare & Gams, 2016). It is very common in serpentine soils (described as *Verticillium leptobactrum* in the past) and in Portugal, it was found in association with bats (Daghino *et al*., 2012; Paiva-Cardoso *et al*., 2014). *Leptobacillium leptobactrum* has been extensively studied because of its potential as biocontrol agent due to its nematicidal capacity against the plant nematode, *Meloidogyne incognita* (Regaieg *et al*., 2011). *Verticillium* species (the genus which *L. leptobactrum* was associated before) were found in marine habitats in association with sponges in Helgoland, Germany (Höller *et al*., 2000). However, there are no records of this fungus being associated with algae in this habitat, which is surprising considering the results of this work, where we observed a high abundance of isolates identified as *Leptobacillium leptobactrum*, mainly distributed in the hosts of site A. In spite of the absence of *Leptobacillium* in marine environments, this genus is included in an order which shelter another fungal species described as secondary inhabitants of marine environment (Kohlmeyer, 1986). A hypothesis for their high abundance in the algae from Ria de Aveiro is related with the strong anthropic influence and their proximity to the terrestrial environment. The human influence can promote the transport of *Leptobacillium leptobactrum* spores from the terrestrial environment (soil) to the marine environment, and this way the establishment of this species in the algae present there. This is the most plausible explanation to justify the strong presence of a fungus typically isolated from soil in a marine habitat, *Leptobacillium* species

The fungal community also included species attributed to the genus *Acremonium* and three related genera: *Emericellopsis, Parasarocladium* and *Sarocladium*. *Parasarocladium* and *Sarocladium* are the only genera well defined in the Sarocladiaceae family, while the remaining genera are polyphyletic due to their multiple ancestries in different families or orders (Giraldo *et al*., 2017; Summerbell *et al*., 2011). *Acremonium*-like species are a widely spread group, with ability to inhabit a sizeable range of substrates and niches, including marine habitats (Grum-Grzhimaylo *et al*., 2013; Giraldo *et al*., 2017; Perdomo *et al*., 2011). The group comprises common soil saprobes, and there are also marine species belonging to *Acremonium* or *Emericellopsis*, which were found associated with algae (Konovalova & Logacheva, 2016). The group of *Acremonium*-like species was also observed in the present survey, where it amounts to 14 % of the overall diversity. *Emericellopsis* species contributed for that value by their strong connection with the algae samples collected from the sites C and E (Figure 4 and 5). Species of *Emericellopsis* are known to occur in different environments: marine, terrestrial and soda soils. For this reason, the occurrence of these fungi in our work isolated from different sites is not surprising, even with distinct salinity levels and variations. *Emericellopsis* species do not require a specific level of salinity to grow, as well as, are capable to tolerate high levels of sodium chloride, which can explain their widespread in algae from Ria de Aveiro (Grum-Grzhimaylo *et al*., 2013; Vaz *et al*., 2005). In addition, the ability to produce compounds with interesting properties, such as antibiotics, has been observed in *Acremonium* related taxa (Carreira *et al*., 2015; Rogozhin *et al*., 2018). In addition to being relevant phytopathogenic fungi, some individuals can cause opportunistic infections in humans (Grum-Grzhimaylo *et al*., 2013; Summerbell *et al*., 2011).

Oliveira and colleagues collected information about fungi community associated with algae. A collection of studies demonstrated the presence of *Penicillium* sp. in *Ulva* sp. and *Epicoccum* sp. in *Fucus* sp. (Oliveira *et al*., 2012). Also, isolates belonging to *Aspergillus* sp. has been detected in brown and red algae and, *Exophiala oligosperma* and *Acremonium* sp. in red algae, as verified in our work. However, the analysis of our fungi collection revealed an interesting result regarding the low abundance of *Aspergillus* sp. in algae communities. In the seaweeds collected in this work, species of this genus had an incidence of only 1.85 % in the overall diversity. This is an unexpected situation because species belonging to this genus usually have a great ability to adapt to different environments (especially the marine) and are dominant in endophytic mycobiota of seaweeds (Raghukumar, 2012), which is denied by the results obtained in this study.

4.3.1. MEASURE OF COMPLEMENTARY OR DISSIMILARITY OF THE FUNGAL COMMUNITIES

To analyse the community composition, it was applied a measure of dissimilarity between pairs of the samples (Ricotta & Podani, 2017). Then, the abundance of each fungal group in the samples was explore using the Bray-Curtis index (Faith *et al*., 1987), which allowed to obtain a "robust" result in "unstandardized data" (Bray & Curtis, 1957).

In order to understand if there was some significant influence of sampling sites, host genera and host phyla in the pairs of comparisons obtained it was applied the Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) analysis. "Host genera" and "algae phyla" did not influence in a significative way the samples [(PERMANOVA: $R^2 = 0.43984$, Pr(>F) = 0.13, Pr(>F) > 0.05) and (PERMANOVA: $R^2 = 0.11589$, Pr(>F) = 0.08, Pr(>F) > 0.05), respectively]. However, the results demonstrated an influence of the sampling sites in the samples communities [PERMANOVA: $R^2 = 0.43101$, Pr(>F) = 0.001, Pr(>F) < 0.05].

TABLE 23 | Results obtained from Adonis analysis.

Number of comparisons	Subjects of comparisons			p.value	p.adjusted
1	A	*vs.*	B	0.00700000	0.147
2	A	*vs.*	C	0.00300000	0.063
3	A	*vs.*	D	0.03000000	0.630
4	A	*vs.*	E	0.01100000	0.231
5	A	*vs.*	F	0.14400000	1.000
6	A	*vs.*	G	0.07200000	1.000
7	B	*vs.*	C	0.31100000	1.000
8	B	*vs.*	D	0.23400000	1.000
9	B	*vs.*	E	0.08900000	1.000
10	B	*vs.*	F	0.72500000	1.000
11	B	*vs.*	G	0.09900000	1.000
12	C	*vs.*	D	0.14400000	1.000
13	C	*vs.*	E	0.60000000	1.000
14	C	*vs.*	F	0.50000000	1.000
15	C	*vs.*	G	0.08800000	1.000
16	D	*vs.*	E	0.06666667	1.000
17	D	*vs.*	F	0.33333333	1.000
18	D	*vs.*	G	0.33333333	1.000
19	E	*vs.*	F	0.40000000	1.000
20	E	*vs.*	G	0.40000000	1.000
21	F	*vs.*	G	0.33333333	1.000

Then, a post-hoc test "Pairwise.adonis" (see supplementary information S4) was performed. The pairwise Adonis analysis (Table 23), without a Bonferroni correction (column of "p.value"), showed four pairs of sampling sites with significant differences (p-value < 0.05): site A differed significantly from sites B, C, D and E. However, after the Bonferroni p-value correction (column of "p.adjusted"), these differences were no longer significative. This happened because Bonferroni correction is very conservative, and consequently, the capacity to detect differences undergoes a great reduction (Narum, 2006).

A Principal Coordinates Analysis (PCoA) was performed based on data obtained from Bray-Curtis distances (Figure 7). Despite the low variance represented in the graphic axes (26.9 % and 14.1 % for PCoA1 and PCoA2, respectively), it is possible to observe a clear separation of the samples from site A in relation to the remain samples distributed. This ecological pattern of the samples supported the differences verified between fungal community of site A and sites B, C, D and E.

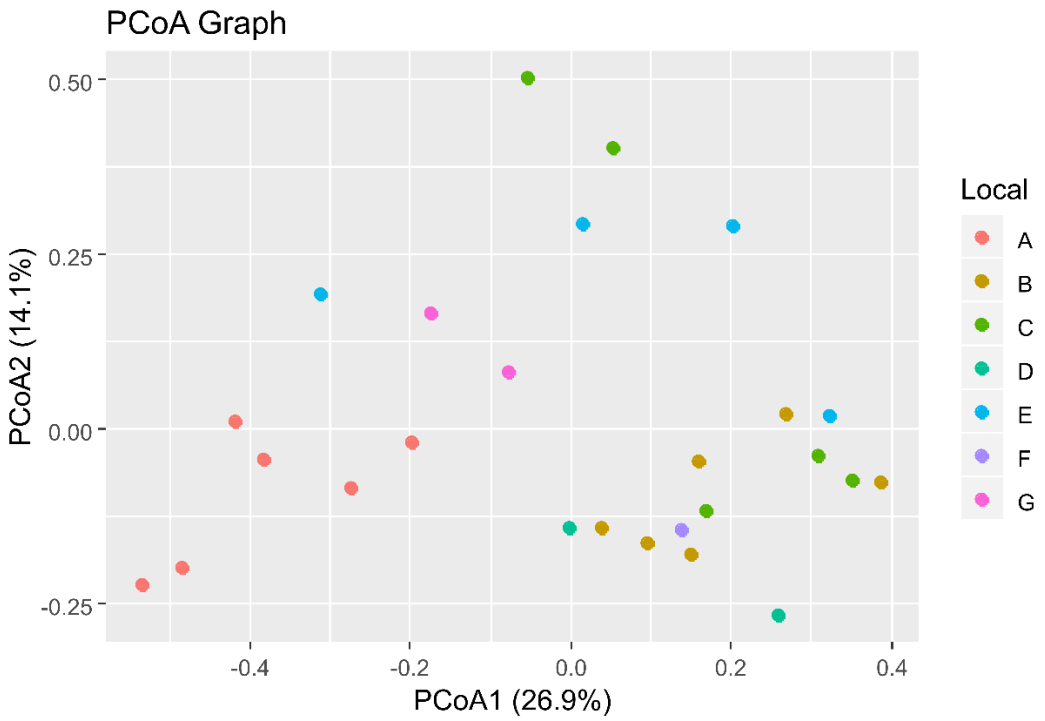

FIGURE 7 | Principal coordinates analysis (PCoA) of the samples used in the current study.

Site A is the one which more differs from the remaining sampling sites (Figure 7). Its proximity with the coast (localized in a marina), places this site under a considerable anthropogenic influence. All the remaining sites are localized further away from the coast and, consequently, suffer a lesser

human-derived impact. This situation, in addition to high salinity (when compared with sites B, C, D and E, which have a very distinct community) can be conditions which promote differences in fungal diversity of the algae. In sites F and G, where salinity reaches similar levels of the salinity in site A, the fungal community does not differ from the site A (Abdel-Gawad *et al*., 2014; Calado *et al*., 2015). As shown by the results of *multipatt* analysis, the two genera which exhibit a preference for the colonization of the algae from site A are *Leptobacillium* and *Paraphaeosphaeria* fungi, where they were found in high abundance. Despite their endophytic characteristic, both fungi groups represent new records in marine environment (Nicot *et al*., 2019) and as algae ihnabitants. Usually, the mycoparasite *Paraphaeosphaeria* and *Leptobacillium* are typically soil inhabitants and/or endophytic of plants (Nicot *et al*., 2019; Zare & Gams, 2016). It is possible that, abiotic factors such as wind and rain, or biotic factors like transition organisms and the humans, be the factors responsible for the dispersion of the respective fungi spores to marine environment. After that, the high salinity and their endophytic characteristic promote the colonization of the algae present in that site. This situation suggests an explanation for the fungal diversity results obtained in the algae collected in this current study.

The analysis of Bray-Curtis index indicated a similar fungal community between the local B and F. These two places are geographically close, which suggests that the fungi distribution between them must be similar or less variable. In addition, the salinity factor can be applied in this situation because the sodium chloride concentrations are similar (Vaz *et al*., 2005). The remaining comparisons between places using the Bray-Curtis index did not demonstrate significant differences of the fungi community assemblages of each local.

These results support the salinity and anthropogenic presence, as the possible influencers which determine the fungal community distribution by algae from Ria de Aveiro, in this survey.

Some authors defend the idea that the geographical localization has a higher impact in fungi community than the host where the organisms are present (Raghukumar, 2012). In addition to salinity and anthropogenic pressures, there are other factors responsible for the fungi distribution in algae. Vegetation composition of the sites (Calado *et al*., 2015; Mohamed & Martiny, 2011) and others environmental stressors (elevated atmospheric CO_2 concentration leading to the increase of acidification and temperature of the seawater) can affect the availability of the substrates and pollution (Loos *et al*., 2019). However, the lack of information about these factors in Ria de Aveiro did not allow the establishment of relation between them and our results.

4.4. CHARACTERIZATION OF PUTATIVE NEW SPECIES

It is known that the current fungal diversity registered only represents a small piece of the entire fungi existing. As mentioned before, algae represent the second biggest source of fungi. However, these organisms are little explored in Portugal. During this survey, it was found six novel species belonging to *Acremonium*-like species: *Emericellopsis cladophorae* sp. nov., *E. enteromorphae* sp. nov., *E. phycophila* sp. nov., *Parasarocladium aestuarinum* sp. nov., *P. alavariense* sp. nov. and *P. fusiforme* sp. nov. (Gonçalves *et al*., 2019[3]).

During the results examination, it was noted a set of isolates which were not discriminated at genus level, indicating the presence of one single species of a putative new genus, belonging to Didymosphaeriaceae family in the Pleosporales order. This putative new genus was described as *Neptunomyces aureus* gen. et sp. nov. (Gonçalves *et al*., 2019[4]).

In addition, it was found one putative new record belonging to the genus *Cladosporium* (*C. rubrum*) and another belonging to *Hypoxylon* group (*H. aveirense*). These two new records description and characterization are presented in the section below.

TAXONOMY

Cladosporium rubrum T. Vicente, M. Gonçalves & A. Alves

Etymology. Name refers to the color of a red pigment produced by this fungus in the culture medium.

Colonies on OA reaching 2.9 cm (ø) after 14 days of incubation at 25 ^{o}C, presenting aurora red and flesh red on the margin of the colony (A; obverse); reverse presenting a scarlet red mixed with vermilion red, with occasional black spots of mycelium; margin slightly irregular. On PDA attaining 2.9 cm, the culture presents a hair brown to greyish white on the border (C and D; obverse and reverse, respectively); On SNA reaching 3.2 cm, the culture is wine yellow to scarlet red on the margin (E and F; obverse and reverse, respectively) with some white mycelium spots on the obverse and dark points on the reverse view.

Mycelium at surface and submerged in culture medium. Hyphae septate, branched, 2.02 – 4.36 µm wide, smooth - and thick-walled, subhyaline to pale brown.

Conidiophores erect, septate, cylindrical, smooth - and thick-walled, subhyaline to "hair brown" in the extremities, presenting occasional nodules, simple or branched, 36.77 – 165.53 µm long and 2.34 – 5.03 µm wide.

Ramoconidia occasional, smooth- and thick-walled, subglobose, 7.08 – 15.72 µm long and 2.55 – 5.55 µm wide, subhyaline to pale brown on the borders. ***Conidia*** obovate to oblong-elliptical, 5.27 – 11.28 µm long and 2.13 – 4.62 µm wide (av. (± SD) 7.78 (±1.33) and (3.18 (± 0.54)), forming chains, smooth- and thick-walled, subhyaline to pale golden with a protuberant and darker conidial hilum.

Cardinal temperatures for growth – Optimum 20^{o} C, Maximum 30^{o} C, minimum 10^{o} C (in PDA).

Specimen examined. Portugal, Aveiro, recovered from an alga (*Ceramium sp.*), Sep.2018.

***Notes*.** A multi-locus analysis placed *Cladosporium rubrum* as a new and distinct species, but closely related to *Cladosporium flavovirens*. Morphologically, these two species are very similar, but *C. flavovirens* presents non-nodulose and longer conidiophores than *C. rubrum*. In addition, there was observed some differences in the size of the fungal structures analyzed for this group: *C. rubrum* has larger hyphae, smaller ramoconidia (long) and bigger conidia than *C. flavovirens*. This new taxonomic group is inserted in the complex Cladosporioides, in a small group well defined, where are included *C. flavovirens* and *C. flabelliforme*.

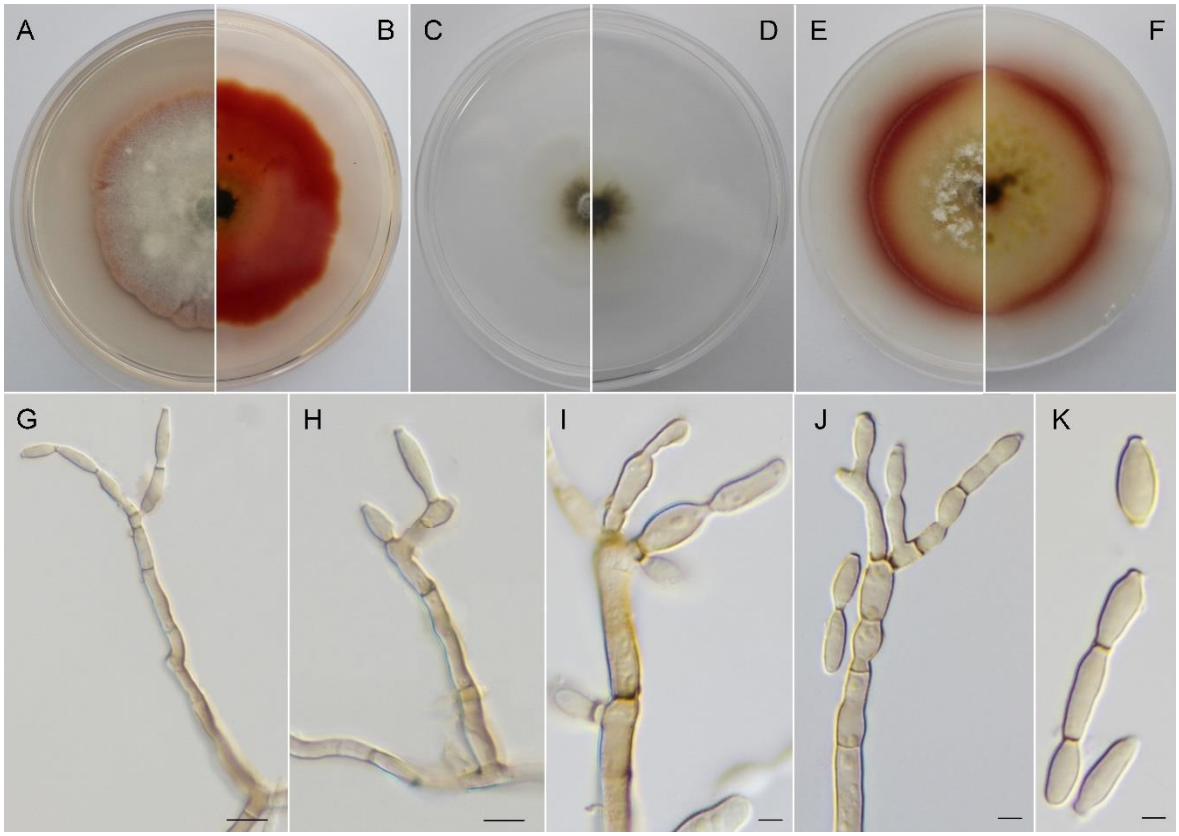

FIGURE 8 | *Cladosporium rubrum*. A, B – Culture in PDA (obvers and reverse view, respectively), after 14 days at 25º C (in the dark). C, D – Culture in SNA (obvers and reverse view, respectively), after 14 days at 25º C (in the dark). E, F – Culture in OA (obvers and reverse view, respectively), after 14 days at 25º C (in the dark). G-I – Conidiophores, ramoconidia and conidia. J - Ramoconidia and conidia chain. K – Conidia chain. Scale bars = 10 µm.

Cladosporium is a very large genus of fungi organized (phylogenetically and morphologically) in three complexes: *C. herbarum*, *C. sphaerospermum* and *C. cladosporioides* (Bensch *et al*., 2012; Bensch *et al*., 2018). *C. rubrum* is included in the last one mentioned: Cladosporioides complex, a well-established group, which is phylogenetically individualized from the remaining complexes (Bensch *et al*., 2012). All the species belonging to this group have similarities with *C. cladosporioides*, presenting slightly morphological differences, but resolved by a polyphasic analysis using a combination between the genes of ITS, actin and translation elongation factor 1-α (Bensch *et al*., 2010; Bensch *et al*., 2018). Since early, these species have demonstrating a huge ecological versatility which allow them to inhabit soil, textiles, indoor/outdoor environments, decaying vegetal matter and possibly involved in human superficial infections (Bensch *et al*., 2010; Bensch *et al*., 2018; Sandoval-Denis *et al*., 2016). The *Cladosporium* species are also usual endophytes in algae (Ding *et al*., 2008; Thirumalanadhuni & Palempalli, 2018; Trivedi *et al.,* 2015).

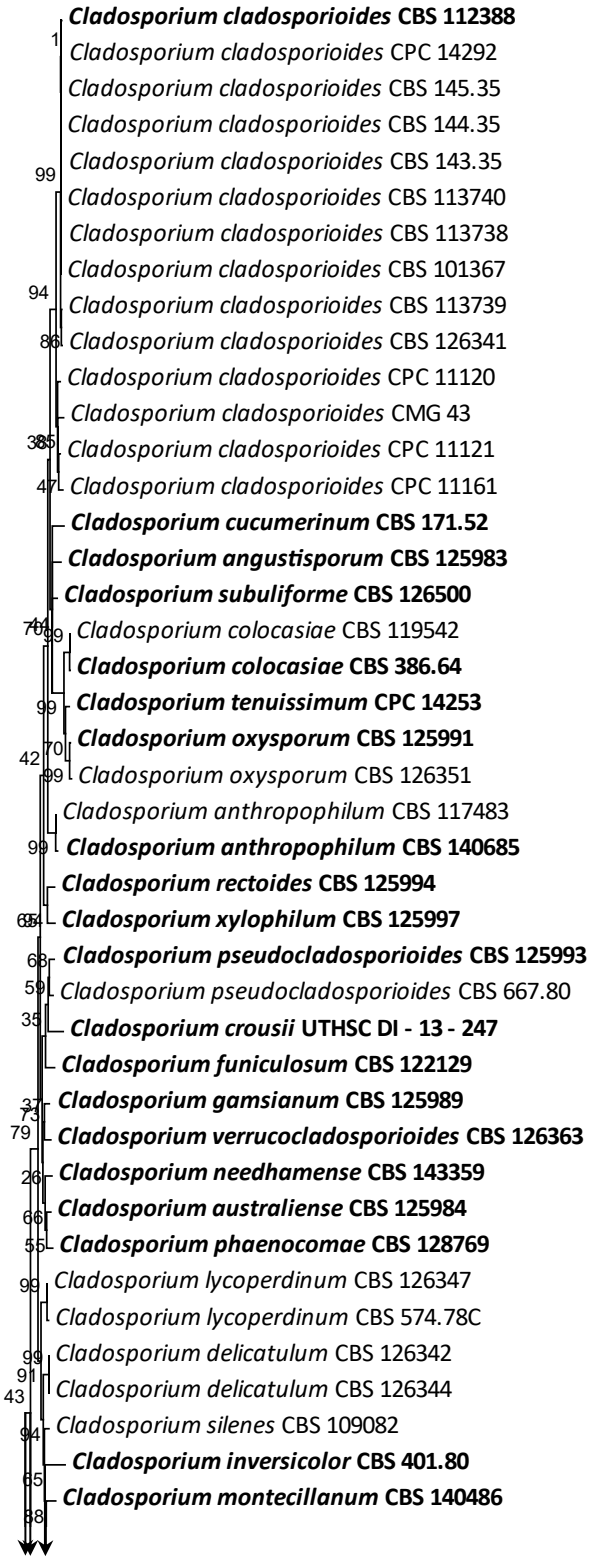

FIGURE 9 | Maximum likelihood (ML) tree obtained from the phylogenetic relationships of *Cladosporium* species (*Cladosporioides* complex) based on combined ITS, actin and translation elongation factor 1-α, under the General Time Reversible model. The tree is rooted with *Cercospora beticola* CBS 116456 and it is drawn to scale, which branches size represent the number of substitutions per site. Bootstrap values are shown at the nodes. All the ex-type species are highlighted in bold and the new species is represented in marine blue.

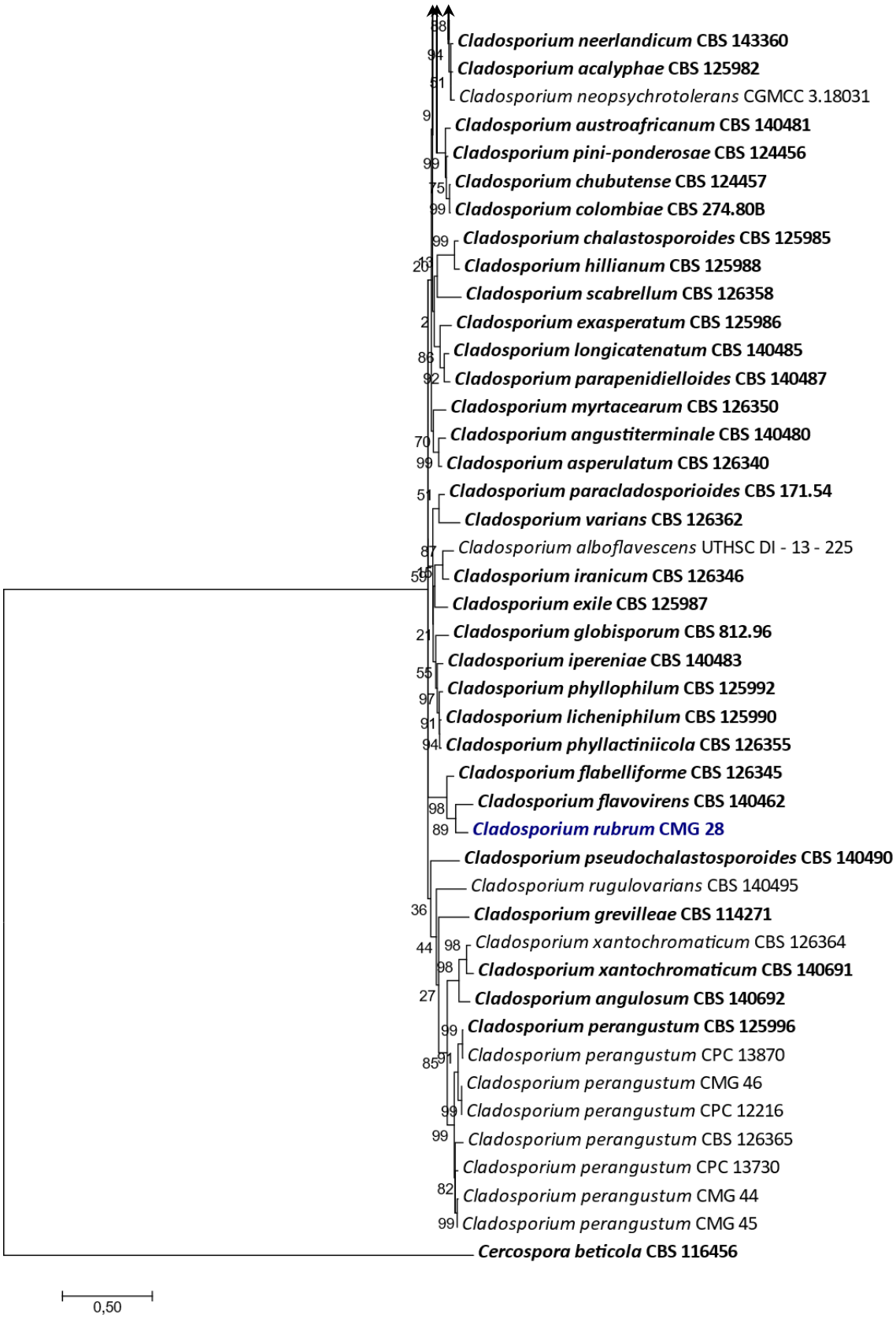

FIGURE 9 | (continued).

Cladosporium species also can be saprobes, cosmopolitan and their conidia have a great "power" of dissemination (Bensch *et al*., 2012). This survey carried out in Ria de Aveiro, adds a new record species to the large group composing the genus *Cladosporium*, but it represents a pioneer investigation of these endophytes' fungi sheltered by algae in this environment.

Hypoxylon aveirense T. Vicente, M. Gonçalves & A. Alves

Etymology. Named after the city where it was recovered for the first time, Aveiro.

Holotype. Portugal, Ria de Aveiro, *Fucus* sp.

Known distribution. Portugal, Ria de Aveiro, algae hostc (current information).

Colony on PDA attaining 3.8 cm, after 7 days of incubation at 25 ^{o}C (in the dark). The colony presenting yellowish grey/skimmed milk white in the margin and hair brown/broccoli brown in the centre (A; obvers) and skimmed milk white in the margin, being yellowish in the middle and asparagus green in the centre of the colony (B; reverse). Colony on OA reaching 2.8 cm after 7 days of incubation at 25 ^{o}C, presenting purplish white (C, D; obvers and reverse).

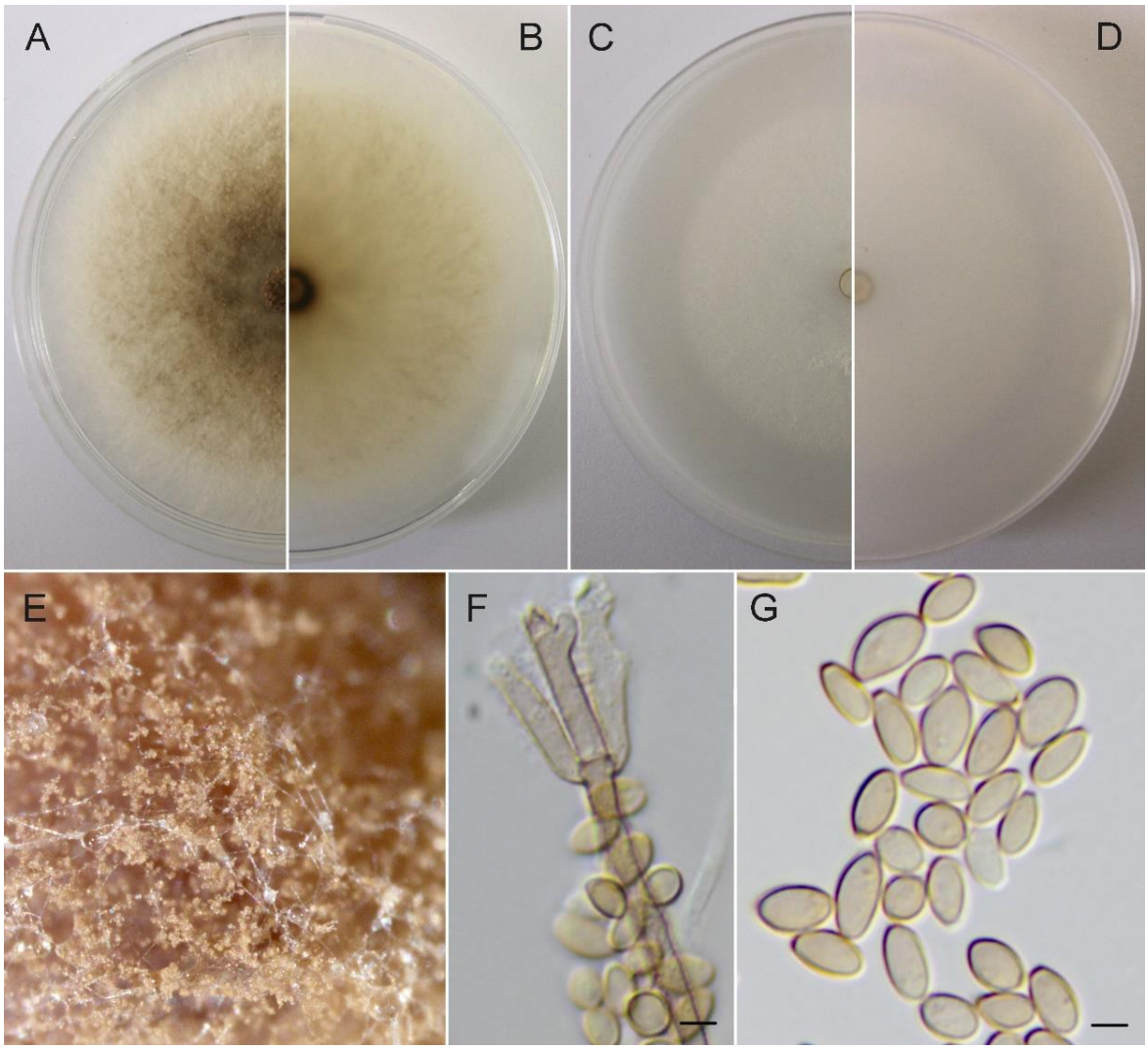

FIGURE 10 | *Hypoxylon aveirense*. A, B – Culture in PDA (obvers and reverse view, respectively), after 7 days at 25º C (in the dark). C, D – Culture in OA (obvers and reverse view, respectively), after 7 days at 25º C (in the dark). E – Close-up view of the culture cultivated in PDA (wood brown). F - Conidiogenous structure nodulisporium-like. G – Conidia. Scale bars = 10 µm.

The conidiogenous structures of the asexual state of *Hypoxylon aveirense* is characterized by a Nodulisporium-like asexual morph. *Conidiophores* are hyaline, pale brown to gold, smooth and thin wall. *Conidiogenous cells* terminal, hyaline and gold color with thin wall, 3.93 – 13.18 × 0.59 – 3.30 µm. *Conidia* ellipsoid or obovate, gold, smooth and thick wall, 3.98 – 6.96 × 2.11 – 3.58 µm.

Cardinal temperatures for growth – Optimum 25º C, Maximum 35º C, minimum 10º C (in PDA). In OA: optimum 30º C, maximum 35º C and minimum 10º C.

Specimen examined. Portugal, Aveiro, recovered from an alga (*Ceramium sp.*), Sep.2018.

Notes. *Hypoxylon aveirense* is phylogenetically isolated from the remaining species reported of this group. The species closest is *H. griseobruneum*, which presenting a vigariella-like structure, different from the nodulisporum-like structure of *H. aveirense* (Kuhnert *et al*., 2014). Other discrepancies are verified in the size of the structures: *H. aveirense* has the conidiogenous cells and conidia smaller than *H. griseobrunneum*. Conidia shapes and colony colours are characterists which place *H. aveirense* close to *H. griseobrunneum*, in addition the combined phylogenetic information in the ITS and β-tubulin sequences.

Hypoxylon is a genus widely disseminated by world, being reported in "Yungas" of Argentina (Sir *et al*., 2016), Martinique (French Caribbean) (Kuhnert *et al*., 2014), France, Portugal and United Kingdom (Fournier *et al*., 2010). In addition, *Hypoxylon* is inserted in a group denominated *Nodulisporium* species, phytopathogens (production of a phytotoxic compound by *Hypoxylon* spp.) and typical endophytes of putrefying vegetal matter. This group only comprises anamorphic fungi and beyond being associated with algae, they have the potential to produce compounds presenting algicidal properties (Ulloa-Benítez *et al*., 2016). *Hypoxylon* is a group already found in Portugal, when *H. lusitanicum* was described in association with wood in aquatic environment (Fournier *et al*., 2010). However, *H. aveirense* is the first report of this genus in algae from Ria de Aveiro. Further studies aiming the chemotaxonomy profile and teleomorphic morphology are necessary to reinforce the phylogenetic placement of this species. In addition, it is interesting the study about the potential of this new species due the possibility of to possess traits as antimicrobial, cytotoxic and insecticidal (Fournier *et al*., 2010).

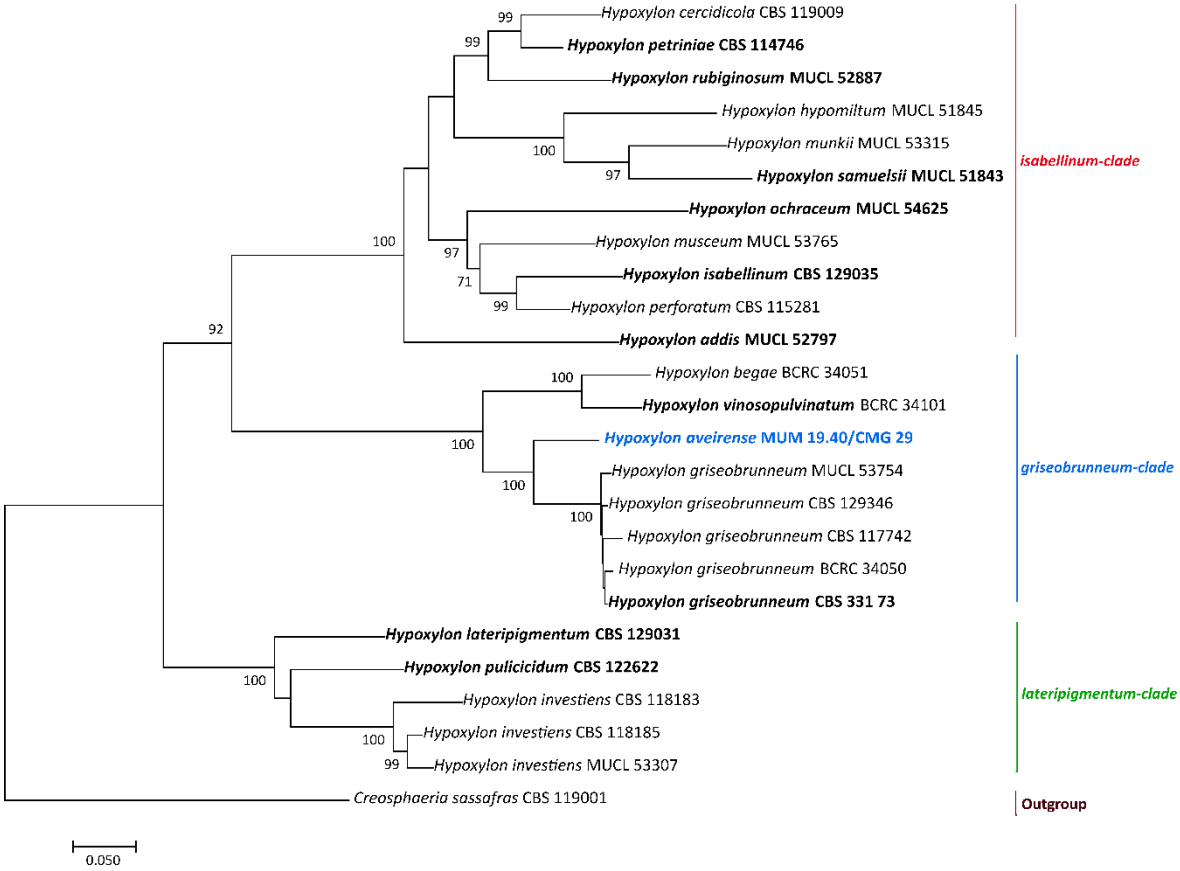

FIGURE 11 | Maximum likelihood (ML) tree obtained from the phylogenetic relationships of *Hypoxylon* species based on combined ITS and β-tubulin genes, under the Kimura two-parameter model. The tree is rooted with *Creosphaeria sassafras* CBS 119001 and it is drawn to scale, which branches size represent the number of substitutions per site. Bootstrap values are shown at the nodes. All the ex-type species are highlighted in bold and the new species is represented in blue. In the right side of the tree is indicate the clades of the species.

5. CONCLUSION

This thesis represents the first report of the fungal diversity of algae collected from Ria de Aveiro. Also, it is important to highlight that this study represents only a small portion of the overall diversity which can inhabit this habitat. Most fungi identified belong to Ascomycota, a very abundant group in marine environment. The absence of some species different of Ascomycota can be related with the incapacity of other fungi, such as Basidiomycota, of inhabit the algae tissues. In addition, the use of methods dependent of fungi culture, the time given for the fungi to growth and the standard temperature used for the incubation can represent barriers for species with a slow growth and/or have preference for other temperatures.

The main fungal groups detected in the seaweeds are marine-derived fungi instead of true marine fungi, as supported by Raghukumar (2012). Our data represents a typical scenario of fungi community because it includes species that accidently reached the marine environment, transients, and facultative marine species, with capacity to grow outside the marine or freshwater habitats (Ólafsson, 2017). Genera as *Cladosporium*, *Penicillium*, *Alternaria* and *Acremonium*-like species are expected groups and their high abundance in this work supported their association with the marine environment. However, this work also introduces uncommon inhabitants' fungi groups of algae, in the marine environment, such as the *Leptobacillium* spp. More studies must be done to ensure this fungal group as an algae colonizer of Ria de Aveiro. Also, the fungal communities of the samples present little differences, mainly between different sampling sites. Such diversity differences and the huge quantity of unexpectable isolates, can be related with the anthropogenic pressure in association with another abiotic factors, such as the salinity of the sampling sites.

In addition, this work allowed the introduction of unidentified fungal species, as the *Cladosporium rubrum* and *Hypoxylon aveirense*. The addition of species never referred in the literature emphasizes the necessity to perform more surveys aiming the algicolous fungal communities in Portugal and, possibly, the discovery of more putative new species.

6. FUTURE WORKS

It is known that the use of methods dependent of fungi culture, limits the microbial diversity found, reducing to only 1 % of the total diversity expected (Goecke & Imhoff, 2017).

To complement this work about fungi diversity from seaweed collected on the Ria de Aveiro it would be advisable to perform methods independent of culture to assess the fungal microorganism not cultivable, but also present in the study subject.

Based on the definition of marine fungi on the "repetitive isolation of the same fungi from the same site", the continuation of this project would be a good support for this work. The useful of future works also is related with the fungi genera of low occurrence in the mycobiota of algae, to define if that fungi have an occasional (accidental) presence or not (Raghukumar, 2017).

This survey contributed to the overall knowledge about fungi present in algae of Ria de Aveiro, Portugal, a study area that should be further explored. Additionally, the potential that these microorganisms can possess should be explored, mainly in association with other organisms, which can amplify these characteristics. The antimicrobial activity against bacteria is a property very important, nowadays, due the increasing of resistance of bacteria against the current antibiotics used. Also, some products produced by fungi exhibited antifungal activities against other pathogenic species. In addition, fungi are also responsible to produce compounds with can help in human disturbances such as anti-allergic, anticoagulant, antidiabetic and antitumoral. All these activities shown, suggest the huge potential of marine fungal microorganisms, already little explored. For this, it is important a continuous work aiming the search by these amazing characteristics.

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8. SUPPLEMENTARY INFORMATION

SUPPLEMENTARY INFORMATION S1. DNA EXTRACTION PROTOCOL (BASED ON MÖLLER *ET AL.*, 1992).

1. Grow fungus on suitable media to ensure adequate development of mycelia;
2. Transfer fungal mycelia to 2.2 mL Eppendorf and add 500 µL TES buffer;
3. Mix boil for 3 minutes and then place on ice water for 10 minutes;
4. Add 10 µL of proteinase K (20 mg/mL);
5. Incubate at 65 ºC for 30 minutes (swirl occasionally):
6. Increase the salt concentration by adding 140 µL 5M NaCl;
7. Add 65 µL 10 % CTAB;
8. Incubate at 65 ºC for 30 minutes (swirl occasionally);
9. Add 1 mL of chloroform: isoamylalcohol (24:1) and mix carefully by inversion for 1 minute;
10. Incubate for 30 minutes at 12 000 rpm at 4 ^{o}C;
11. Transfer supernatant (800 µL) to a new 1.5 mL tube;
12. Add 225 µL 5 M NH_4OAc and mix carefully;
13. Incubate for 30 minutes on ice water;
14. Centrifuge for 10 minutes at 12 000 rpm at 4 ^{o}C;
15. Transfer supernatant (1 000 µL) to a new tube;
16. Add 0.55 volume ice-cold isopropanol (500 µL) and mix carefully;
17. Incubate directly for 10 minutes at 12 000 rpm at 4 ^{o}C;
18. Discard the supernatant;
19. Wash 2X with 1 000 µL ice-cold 76 % ethanol (centrifuge for 10 minutes, discard supernatant);
20. Dry DNA pellet on bench;
21. Dissolve pellet in 30 µL – 100 µL TE buffer + RNase (1.5 µL of 10 mg/mL plus 48.5 µL TE buffer). Store DNA dilutions at 4 or -20 ^{o}C and original stocks at -20 ^{o}C.

SUPPLEMENTARY INFORMATION S2. DNA PURIFICATION PROTOCOL.

Protocol for PCR clean-up or DNA purification from enzymatic reactions (manufacturer instructions: NZYTech)

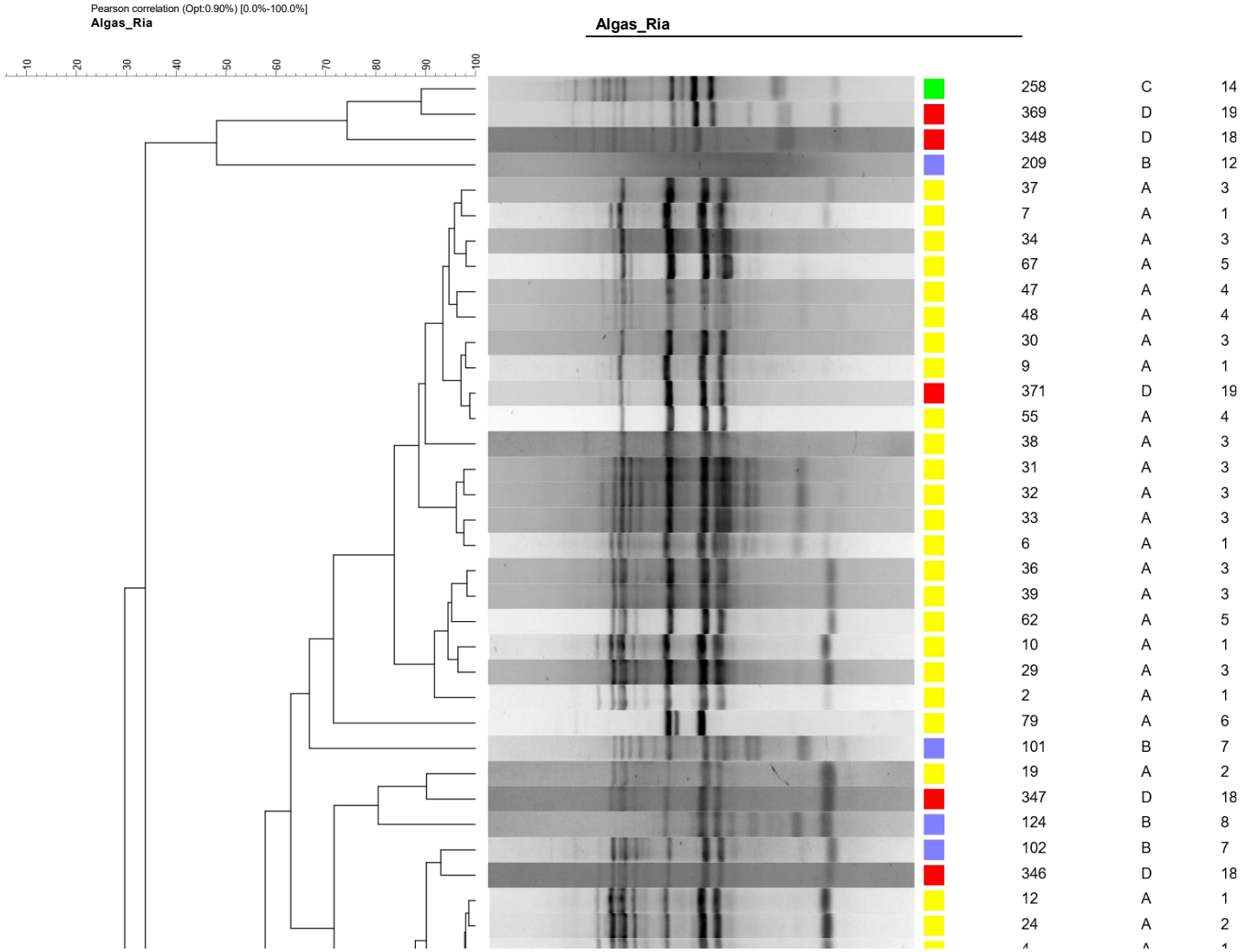
1. Transfer the volume of the reaction mixture into a 1.5 mL microcentrifuge tube and add five volumes of Binding Buffer. Mix by inverting the tube a few times. Centrifuge briefly to collect the sample. All purification steps including centrifugation should be carried out at room temperature.

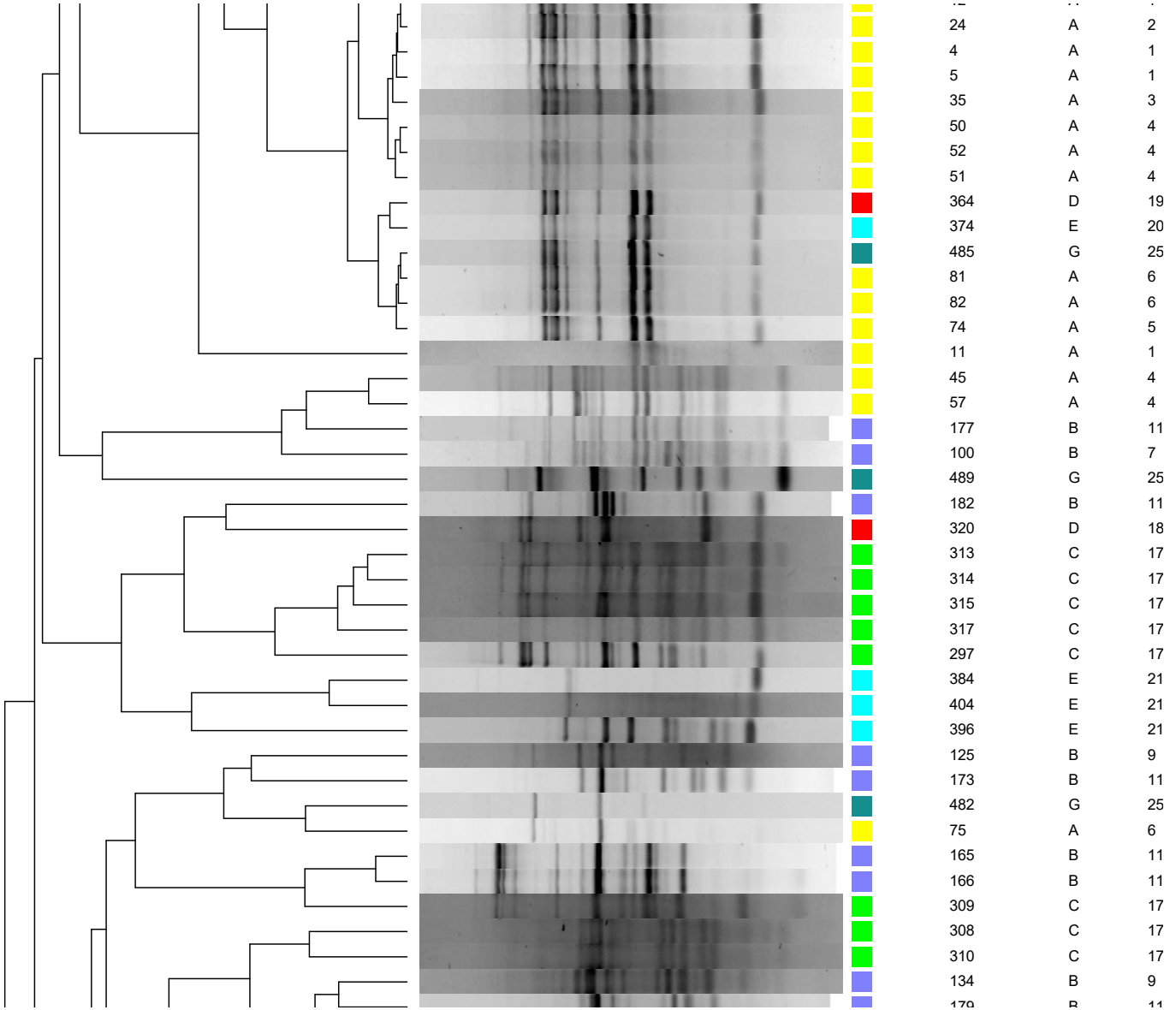
   Note (sequencing of PCR products):

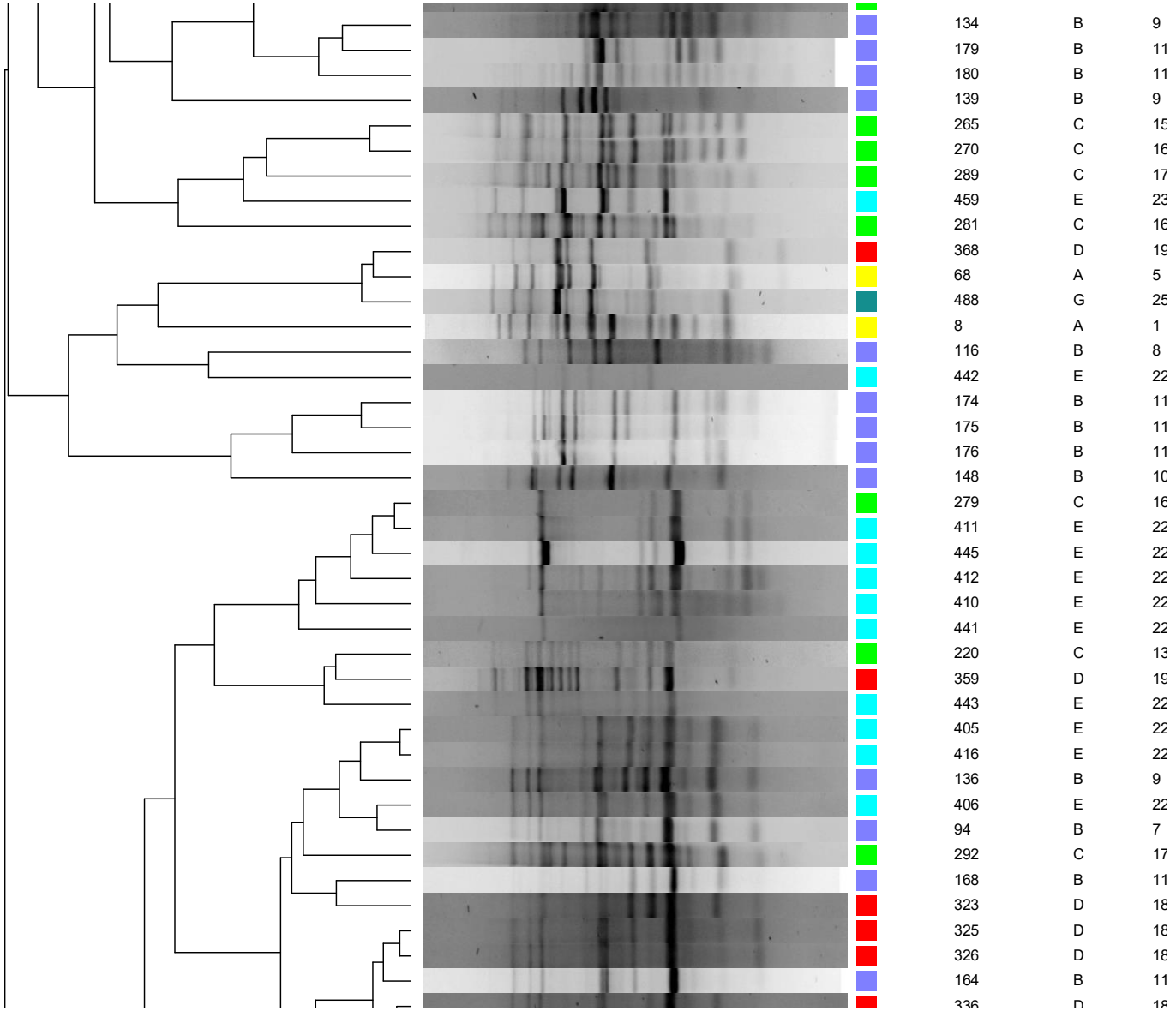
   When cleaning up PCR products for subsequent sequencing processes, if using an amplification primer for sequencing avoid primers larger than 22-25 bp. In this case it is recommended to use a maximum of 0.25 µM of primers during the PCR amplification and 2.5 µM of the sequencing primer in the sequencing reaction. In case residual peaks appear in sequencing chromatograms, due to the presence of traces of the second amplification primer, dilute Binding Buffer to a 60-50 % solution in water and proceed as above. Use NZYTech sequencing services for maximum efficiencies.

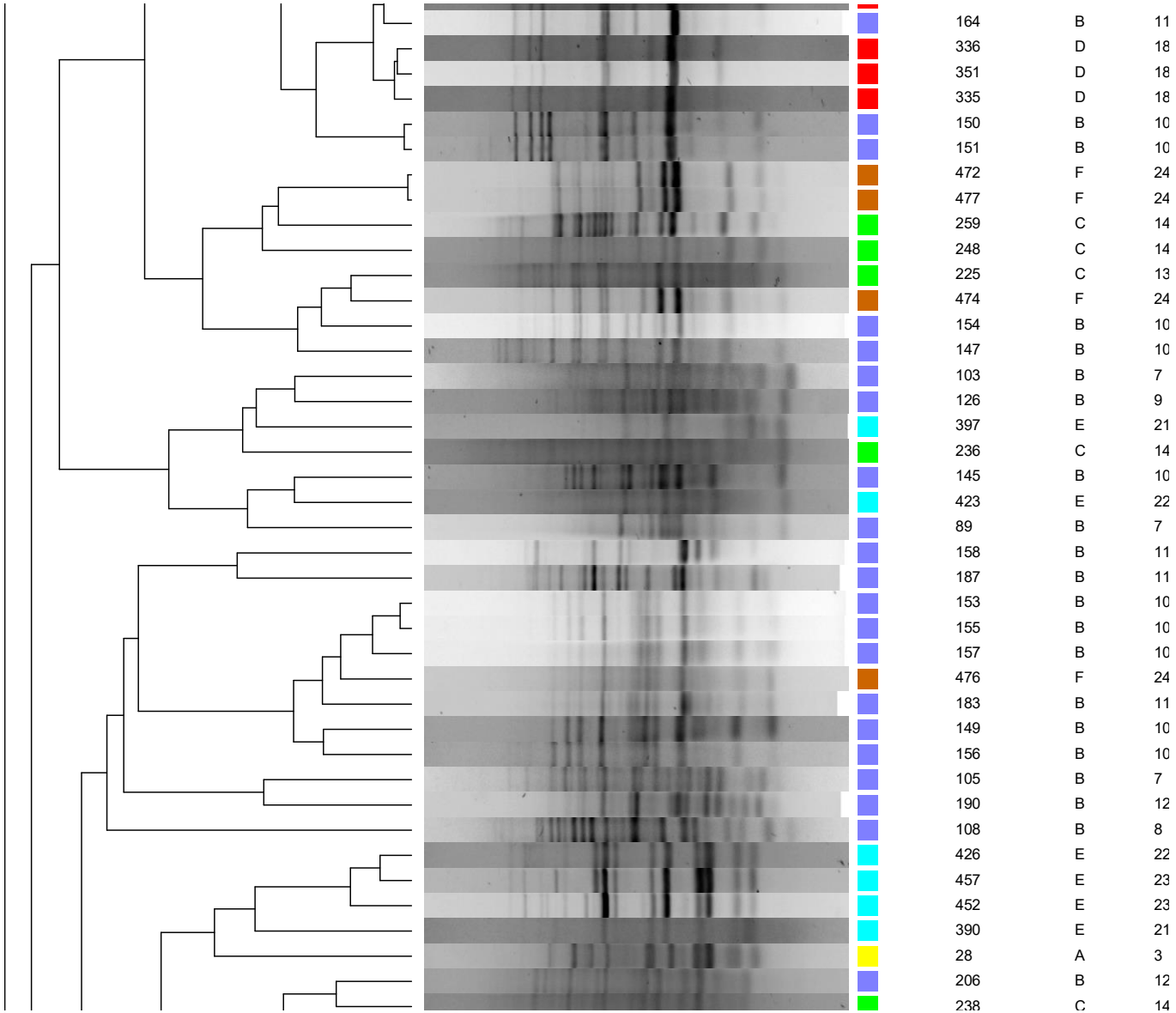
2. Add the above mixture to the NZYTech spin column. The maximum loading volume of the column is 700 µL. For sample volumes greater than 700 µL simply load again. Centrifuge for 30 s to 1 minute and discard the flow-through in the tube.
3. Add 600 µL of Wash Buffer and centrifuge for 30 s to 1 minute. Discard the flow-through in the collection tube.
4. Centrifuge for 1 minute to dry NZYTech spin membrane of residual ethanol.
5. Place the NZYTech spin column into a clean 1.5 mL microcentrifuge tube. Add 50 µL of Elution Buffer to the centre of the column and incubate at room temperature for 1 minute. Centrifuge for 1 minute to elute DNA. Ultrapure water may be used in place of elution buffer. However, DNA recovery with acidic waters may be significantly reduced.
6. Centrifuge for 1 minute to elute the DNA.
7. Store the purified DNA at -20 ^{o}C.

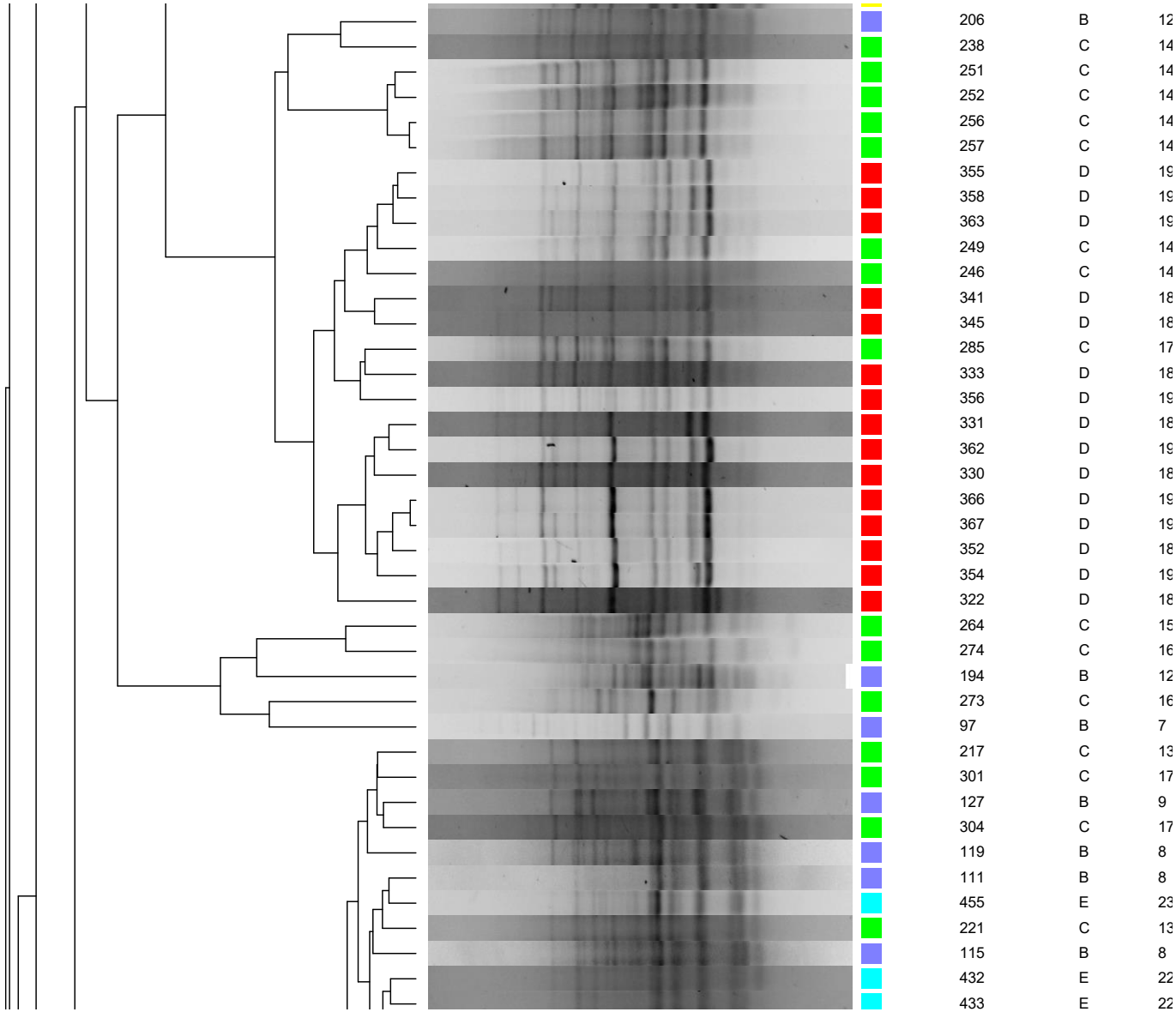
SUPPLEMENTARY FIGURE S3. RESULT FROM MOLECULAR TYPING.

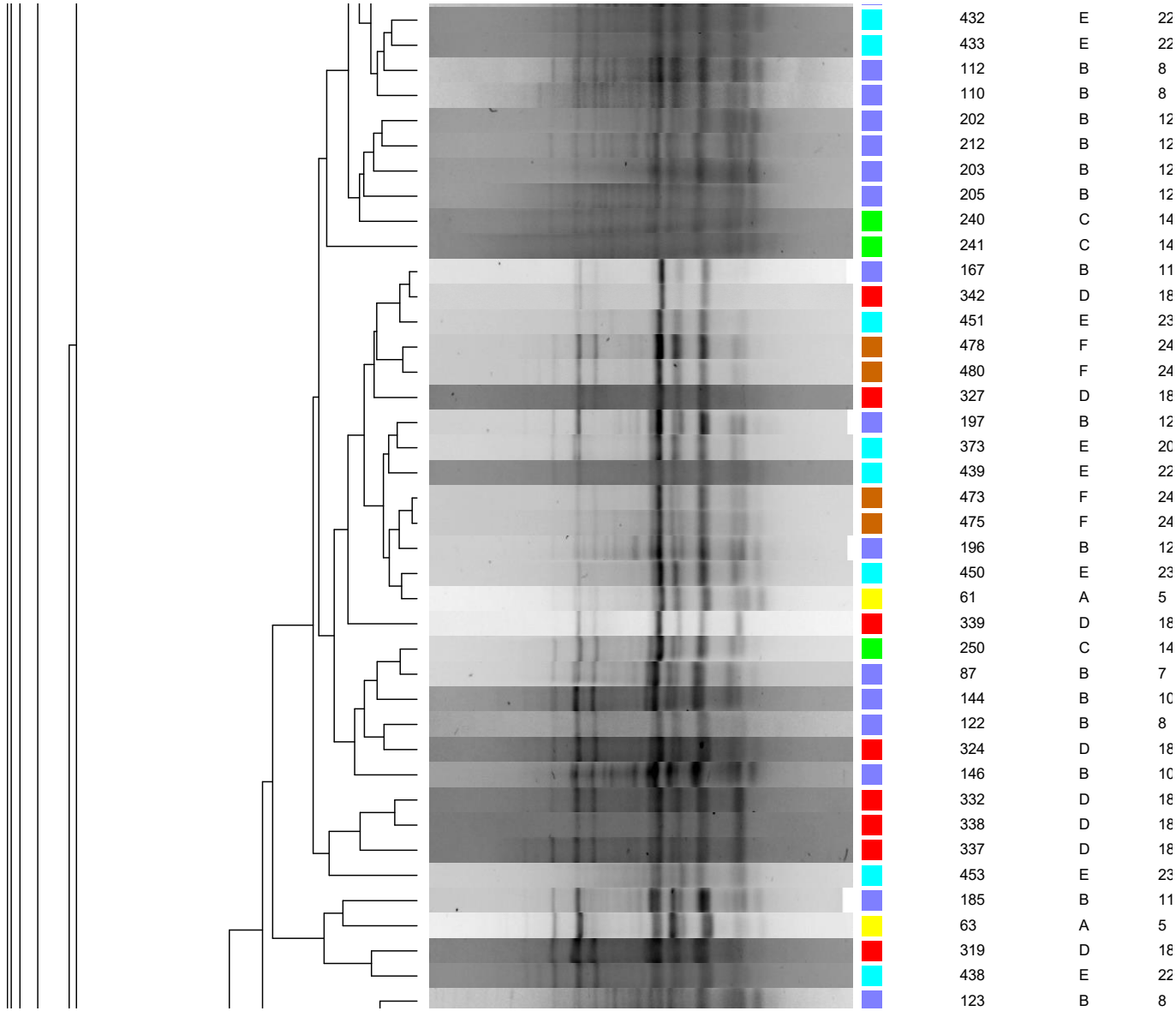


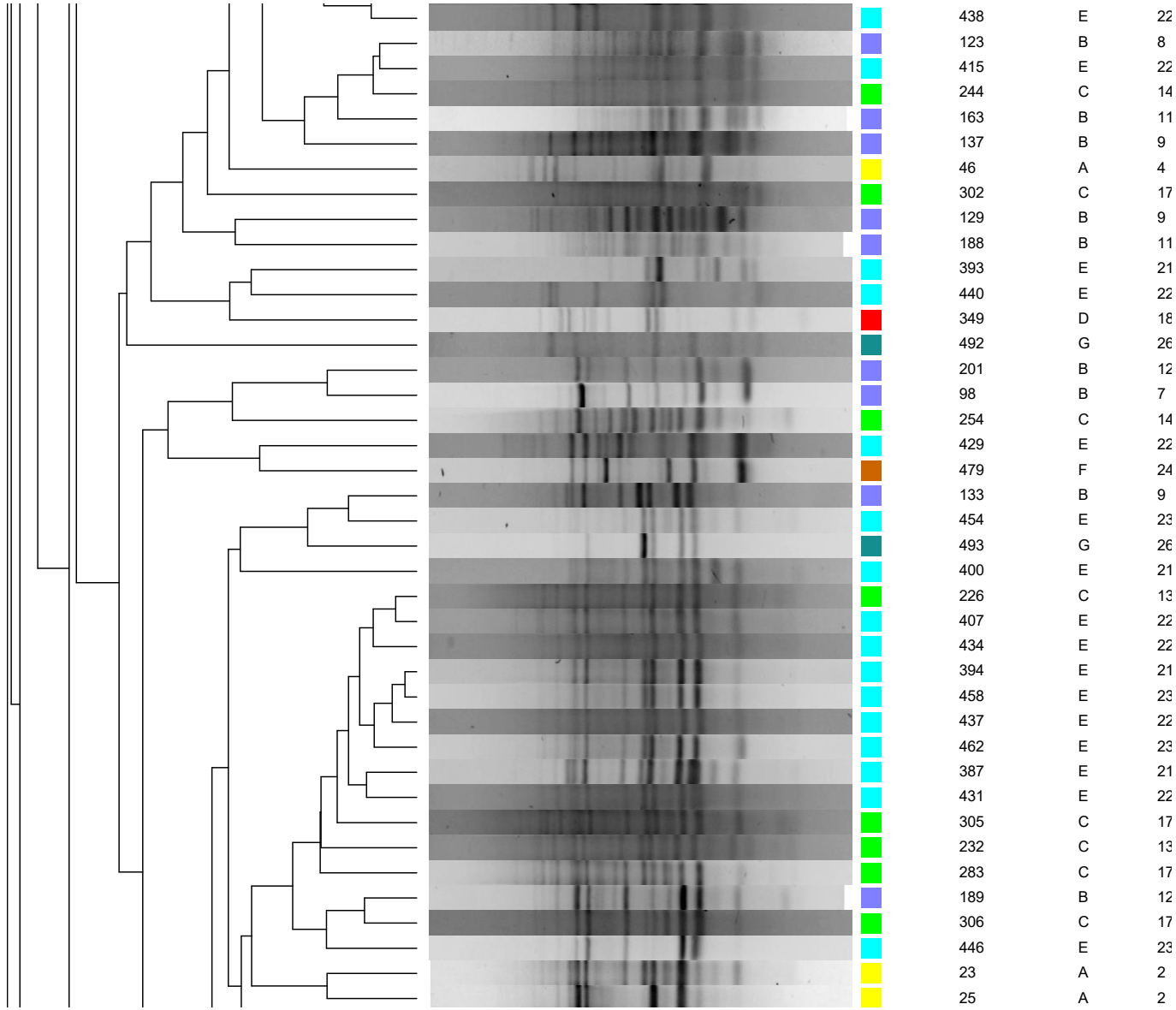

24 A 2
4 A 1
5 A 1
35 A 3
50 A 4
52 A 4
51 A 4
364 D 19
374 E 20
485 G 25
81 A 6
82 A 6
74 A 5
11 A 1
45 A 4
57 A 4
177 B 11
100 B 7
489 G 25
182 B 11
320 D 18
313 C 17
314 C 17
315 C 17
317 C 17
297 C 17
384 E 21
404 E 21
396 E 21
125 B 9
173 B 11
482 G 25
75 A 6
165 B 11
166 B 11
309 C 17
308 C 17
310 C 17
134 B 9


134 B 9
179 B 11
180 B 11
139 B 9
265 C 15
270 C 16
289 C 17
459 E 23
281 C 16
368 D 19
68 A 5
488 G 25
8 A 1
116 B 8
442 E 22
174 B 11
175 B 11
176 B 11
148 B 10
279 C 16
411 E 22
445 E 22
412 E 22
410 E 22
441 E 22
220 C 13
359 D 19
443 E 22
405 E 22
416 E 22
136 B 9
406 E 22
94 B 7
292 C 17
168 B 11
323 D 18
325 D 18
326 D 18
164 B 11


164 B 11
336 D 18
351 D 18
335 D 18
150 B 10
151 B 10
472 F 24
477 F 24
259 C 14
248 C 14
225 C 13
474 F 24
154 B 10
147 B 10
103 B 7
126 B 9
397 E 21
236 C 14
145 B 10
423 E 22
89 B 7
158 B 11
187 B 11
153 B 10
155 B 10
157 B 10
476 F 24
183 B 11
149 B 10
156 B 10
105 B 7
190 B 12
108 B 8
426 E 22
457 E 23
452 E 23
390 E 21
28 A 3
206 B 12
238 C 14


206 B 12
238 C 14
251 C 14
252 C 14
256 C 14
257 C 14
355 D 19
358 D 19
363 D 19
249 C 14
246 C 14
341 D 18
345 D 18
285 C 17
333 D 18
356 D 19
331 D 18
362 D 19
330 D 18
366 D 19
367 D 19
352 D 18
354 D 19
322 D 18
264 C 15
274 C 16
194 B 12
273 C 16
97 B 7
217 C 13
301 C 17
127 B 9
304 C 17
119 B 8
111 B 8
455 E 23
221 C 13
115 B 8
432 E 22
433 E 22


432 E 22
433 E 22
112 B 8
110 B 8
202 B 12
212 B 12
203 B 12
205 B 12
240 C 14
241 C 14
167 B 11
342 D 18
451 E 23
478 F 24
480 F 24
327 D 18
197 B 12
373 E 20
439 E 22
473 F 24
475 F 24
196 B 12
450 E 23
61 A 5
339 D 18
250 C 14
87 B 7
144 B 10
122 B 8
324 D 18
146 B 10
332 D 18
338 D 18
337 D 18
453 E 23
185 B 11
63 A 5
319 D 18
438 E 22
123 B 8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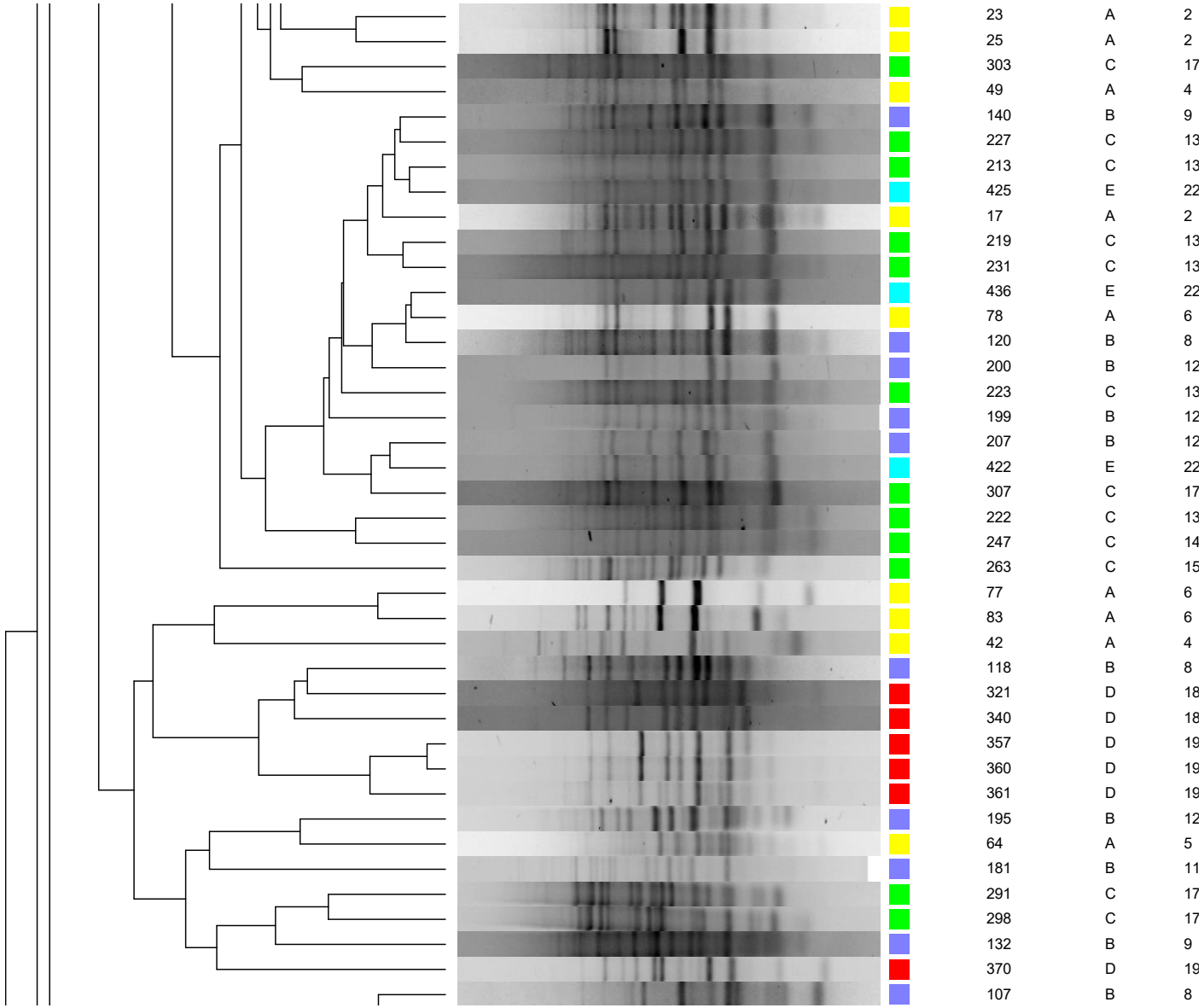
123 B 8
415 E 22
244 C 14
163 B 11
137 B 9
46 A 4
302 C 17
129 B 9
188 B 11
393 E 21
440 E 22
349 D 18
492 G 26
201 B 12
98 B 7
254 C 14
429 E 22
479 F 24
133 B 9
454 E 23
493 G 26
400 E 21
226 C 13
407 E 22
434 E 22
394 E 21
458 E 23
437 E 22
462 E 23
387 E 21
431 E 22
305 C 17
232 C 13
283 C 17
189 B 12
306 C 17
446 E 23
23 A 2
25 A 2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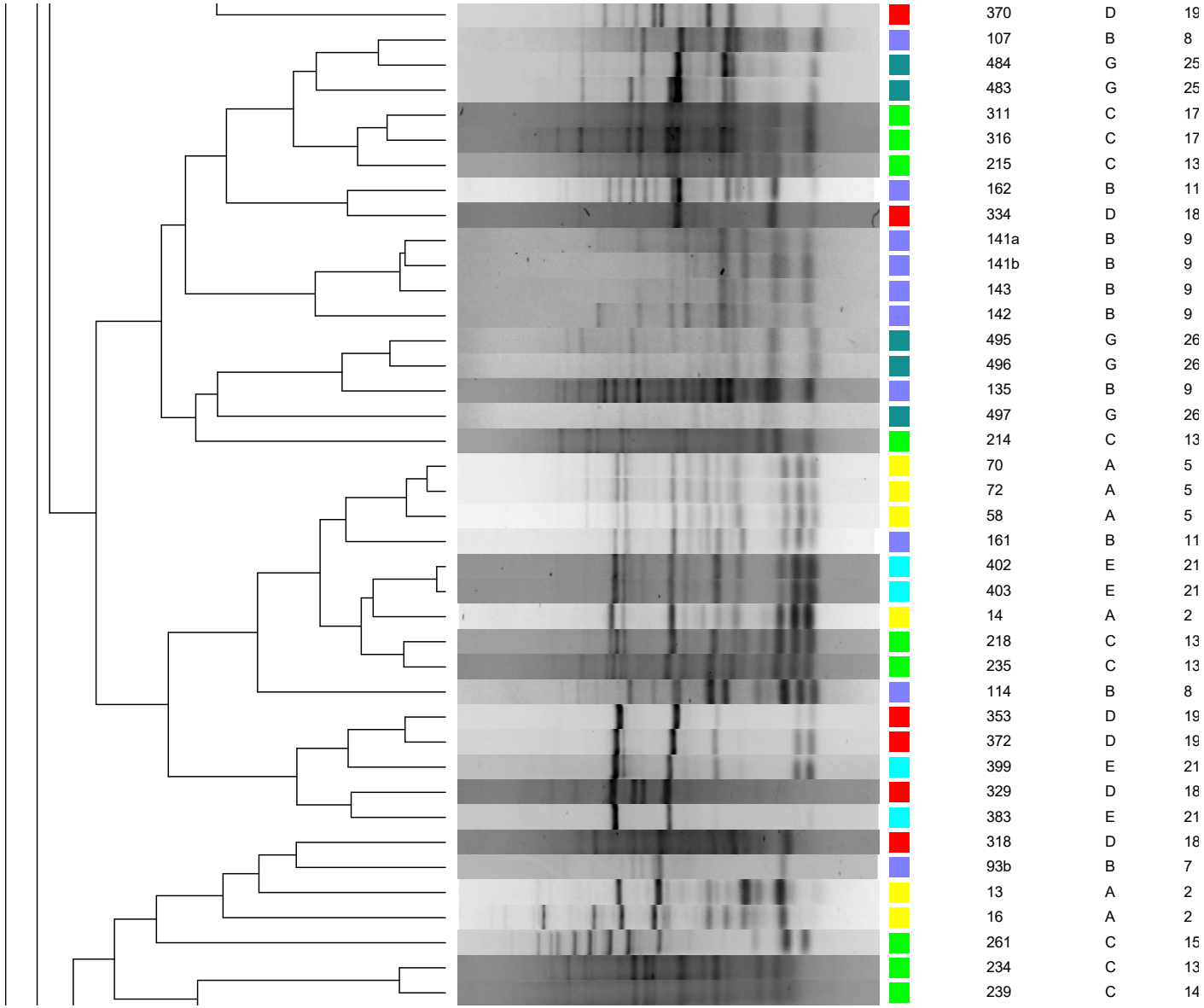
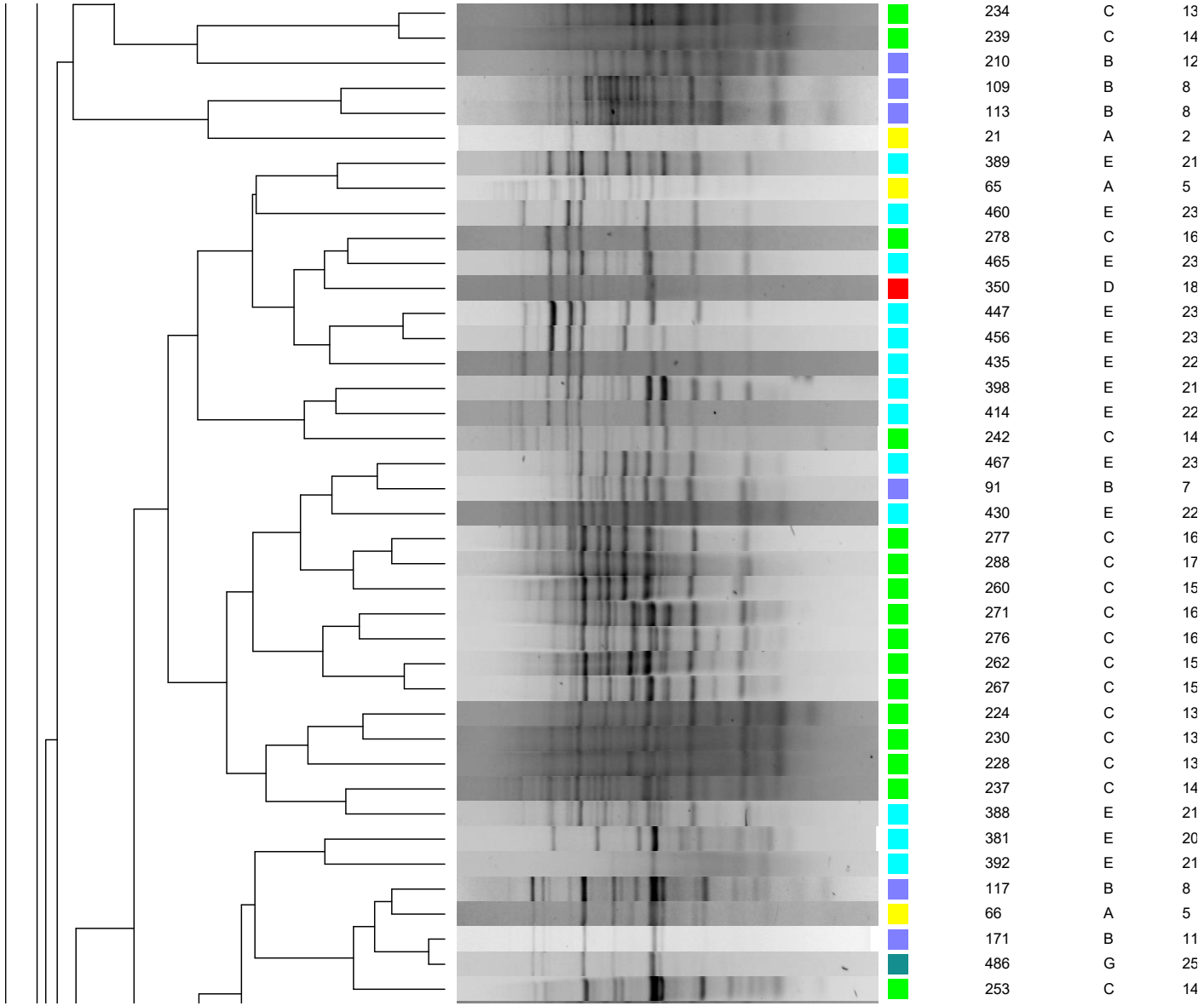
25 A 2
303 C 17
49 A 4
140 B 9
227 C 13
213 C 13
425 E 22
17 A 2
219 C 13
231 C 13
436 E 22
78 A 6
120 B 8
200 B 12
223 C 13
199 B 12
207 B 12
422 E 22
307 C 17
222 C 13
247 C 14
263 C 15
77 A 6
83 A 6
42 A 4
118 B 8
321 D 18
340 D 18
357 D 19
360 D 19
361 D 19
195 B 12
64 A 5
181 B 11
291 C 17
298 C 17
132 B 9
370 D 19
107 B 8


370 D 19
107 B 8
484 G 25
483 G 25
311 C 17
316 C 17
215 C 13
162 B 11
334 D 18
141a B 9
141b B 9
143 B 9
142 B 9
495 G 26
496 G 26
135 B 9
497 G 26
214 C 13
70 A 5
72 A 5
58 A 5
161 B 11
402 E 21
403 E 21
14 A 2
218 C 13
235 C 13
114 B 8
353 D 19
372 D 19
399 E 21
329 D 18
383 E 21
318 D 18
93b B 7
13 A 2
16 A 2
261 C 15
234 C 13
239 C 14


234 C 13
239 C 14
210 B 12
109 B 8
113 B 8
21 A 2
389 E 21
65 A 5
460 E 23
278 C 16
465 E 23
350 D 18
447 E 23
456 E 23
435 E 22
398 E 21
414 E 22
242 C 14
467 E 23
91 B 7
430 E 22
277 C 16
288 C 17
260 C 15
271 C 16
276 C 16
262 C 15
267 C 15
224 C 13
230 C 13
228 C 13
237 C 14
388 E 21
381 E 20
392 E 21
117 B 8
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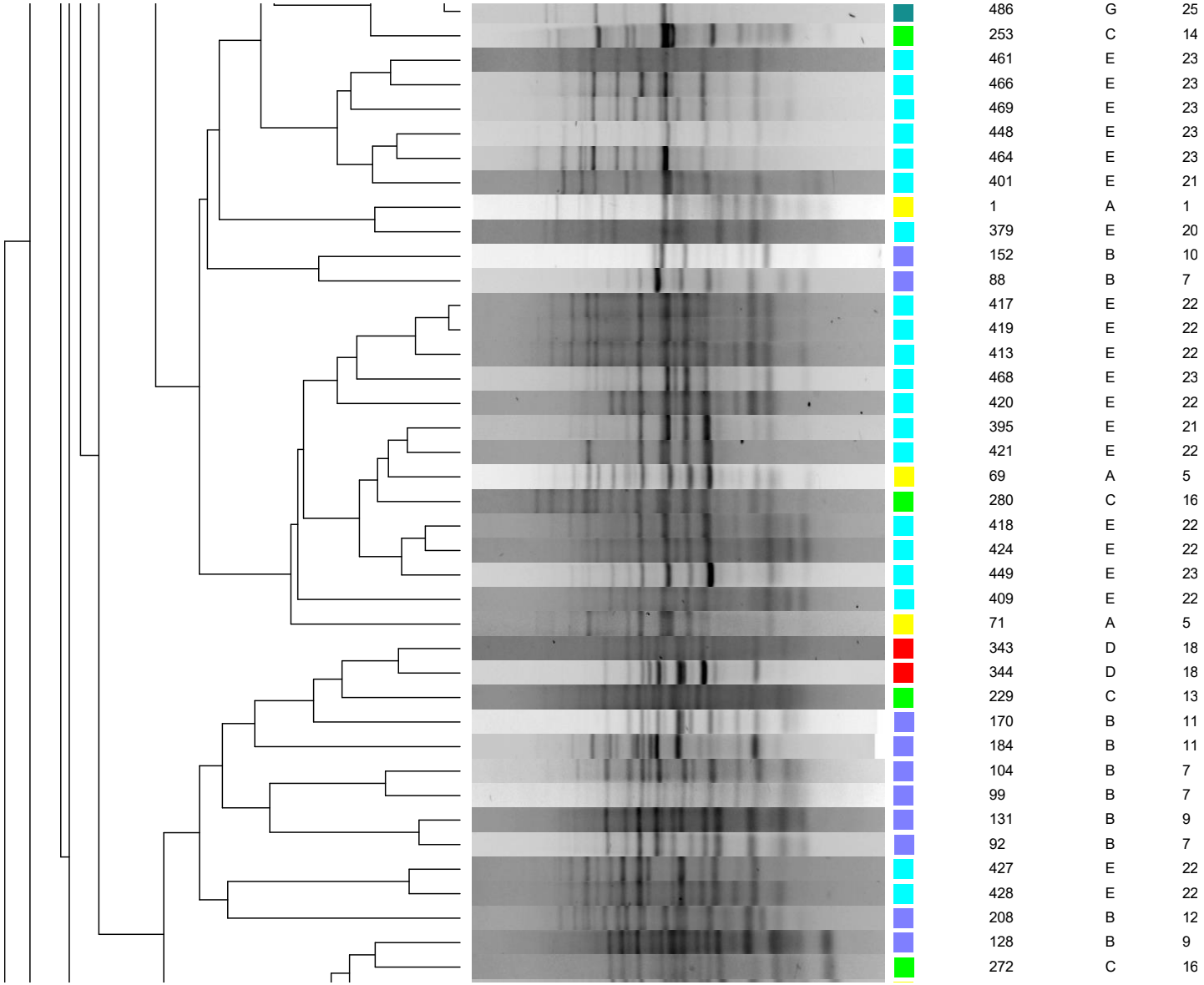
171 B 11
486 G 25
253 C 14


486 G 25
253 C 14
461 E 23
466 E 23
469 E 23
448 E 23
464 E 23
401 E 21
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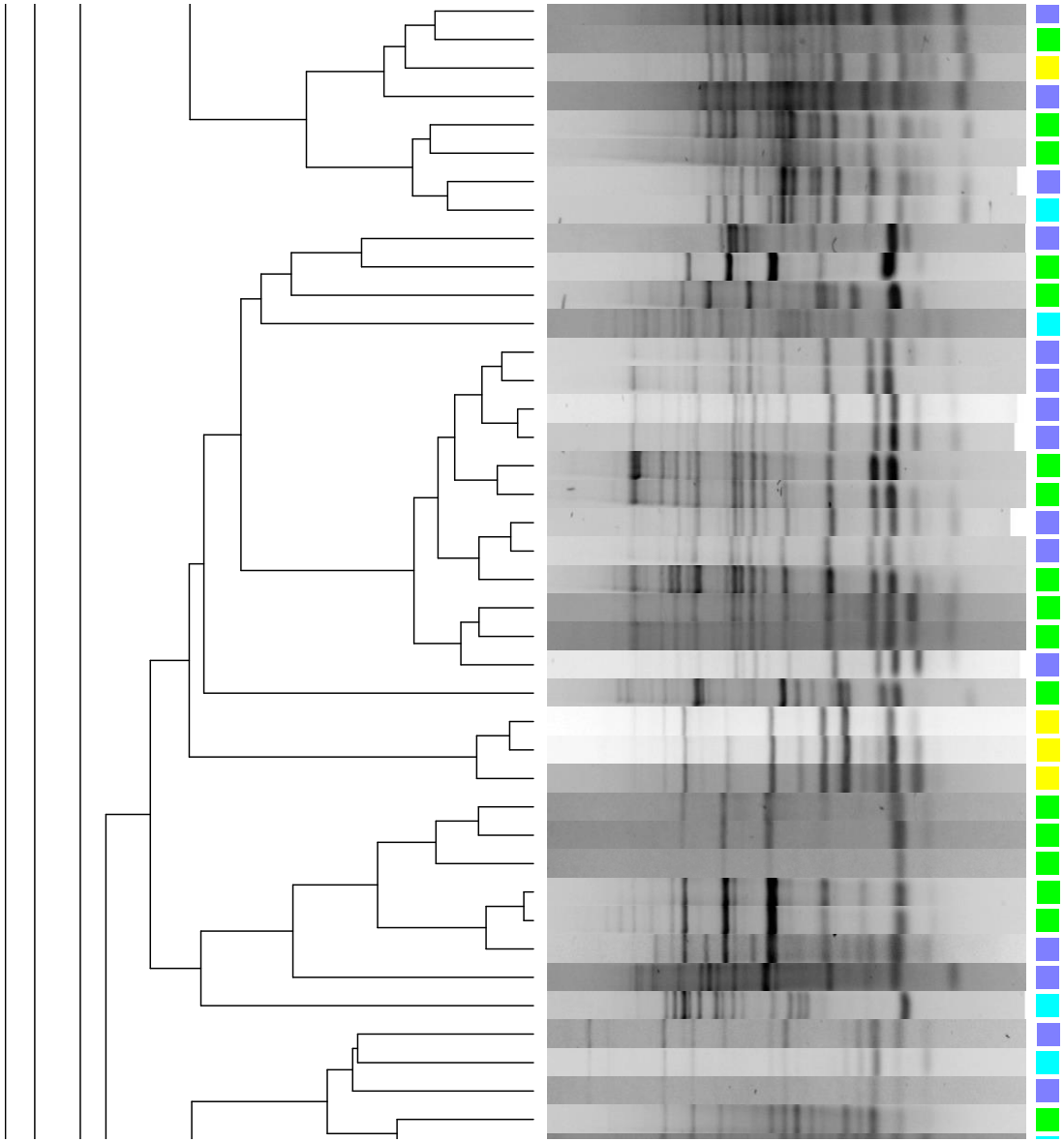
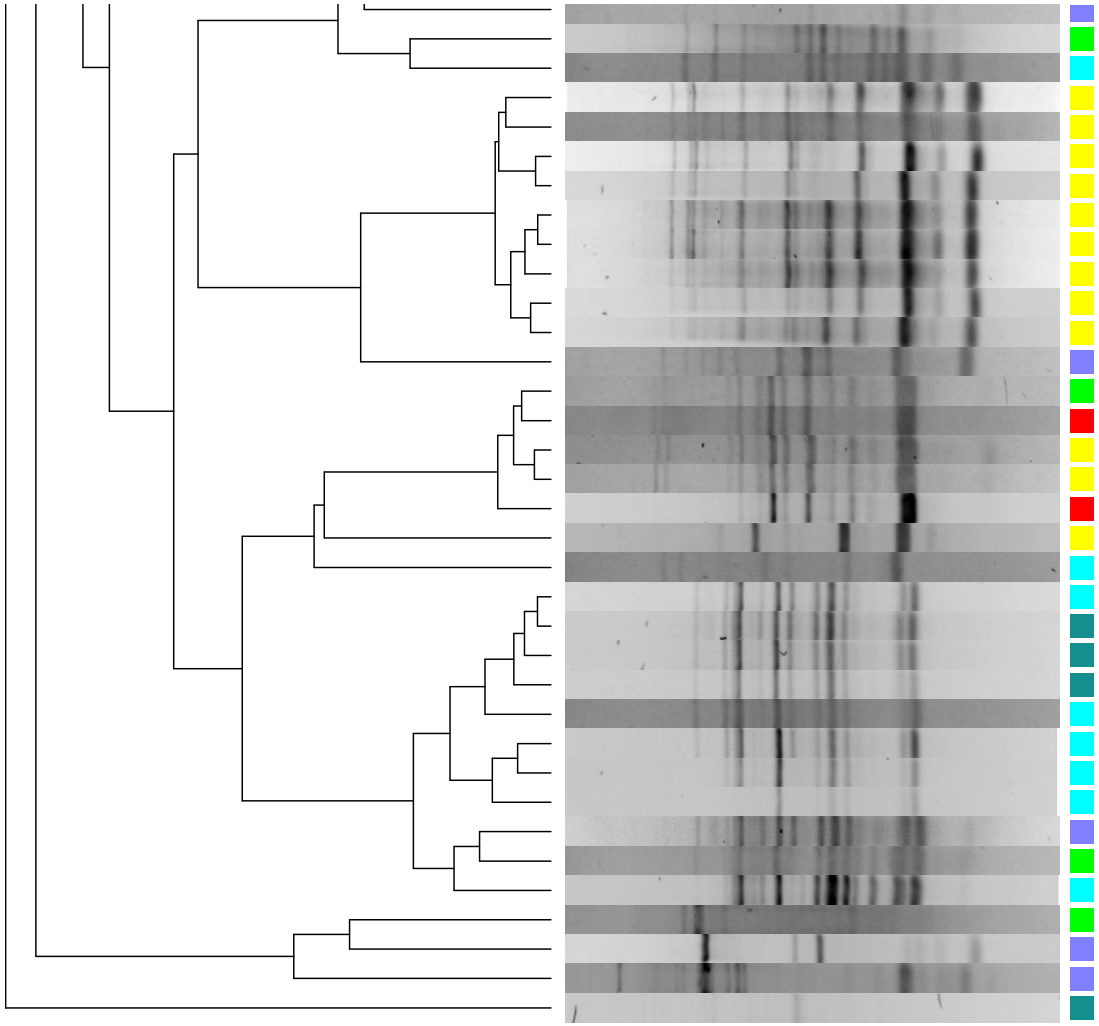
379 E 20
152 B 10
88 B 7
417 E 22
419 E 22
413 E 22
468 E 23
420 E 22
395 E 21
421 E 22
69 A 5
280 C 16
418 E 22
424 E 22
449 E 23
409 E 22
71 A 5
343 D 18
344 D 18
229 C 13
170 B 11
184 B 11
104 B 7
99 B 7
131 B 9
92 B 7
427 E 22
428 E 22
208 B 12
128 B 9
272 C 16



128	B	9
272	C	16
43	A	4
130	B	9
290	C	17
299	C	17
191	B	12
470	E	23
198b	B	12
266	C	15
296	C	17
408	E	22
90	B	7
95	B	7
172	B	11
186	B	11
295	C	17
300	C	17
178	B	11
96	B	7
294	C	17
216	C	13
243	C	14
160	B	11
293	C	17
54	A	4
56	A	4
40	A	4
269	C	15
284	C	17
268	C	15
286	C	17
287	C	17
121	B	8
138	B	9
385	E	21
193	B	12
463	E	23
192	B	12
282	C	16



192	B	12
282	C	16
471	E	23
3	A	1
53	A	4
73	A	5
84	A	6
18	A	2
20	A	2
22	A	2
80	A	6
85	A	6
211	B	12
245	C	14
328	D	18
44	A	4
76	A	6
365	D	19
41	A	4
391	E	21
375	E	20
491	G	26
490	G	26
494	G	26
378	E	20
376	E	20
380	E	20
377	E	20
106	B	7
255	C	14
382	E	20
275	C	16
86	B	7
204	B	12
481	G	25

SUPPLEMENTARY INFORMATION S4. FUNCTION *PAIRWISE.ADONIS*.

```
pairwise.adonis <- function(x,factors, sim.method = 'bray', p.adjust.m ='bonferroni')
{
 library(vegan)
 co = combn(unique(factors),2)
 pairs = c()
 F.Model =c()
 R2 = c()
 p.value = c()

 for(elem in 1:ncol(co)){
  ad = adonis(x[factors %in% c(co[1,elem],co[2,elem]),] ~ factors[factors %in%
c(co[1,elem],co[2,elem])] , method =sim.method);
  pairs = c(pairs,paste(co[1,elem],'vs',co[2,elem]));
  F.Model =c(F.Model,ad$aov.tab[1,4]);
  R2 = c(R2,ad$aov.tab[1,5]);
  p.value = c(p.value,ad$aov.tab[1,6])
 }
 p.adjusted = p.adjust(p.value,method=p.adjust.m)
 pairw.res = data.frame(pairs,F.Model,R2,p.value,p.adjusted)
 return(pairw.res)
 }
```